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Biochemical Characterization of Antibacterial Actinomycetes Isolated from Soils of Hadhramout- Yemen

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CONTENTS

CHAPTER ONE INTRODUCTION

1. Introduction
   1.5 Actinomycetes
   1. 6 Scope
2 Aims and Objectives

CHAPTER TWO: REVIEW OF LITERATURE

2.1 Non - streptomycetes from special habitats
   2.1.1 Caves as special habitats
   2.1.2 Dams as special habitats
   2.1.3 Mountains as special habitats

CHAPTER THREE: RESULTS

3.1 Isolation of actinomycetes from some dams, caves and mountain soils
   3.1.1 Physicochemical characteristics of the samples
3.2 Screening of actinomycete isolates for antibacterial and enzyme activities
   3.1.1 Screening of actinomycete isolates for antibacterial activity
   3.3.2 Antibacterial activity on different media
   3.3.3 Screening of actinomycete isolates for enzyme activities
3.3 Taxonomy of actinomycetes
3.4 Study on the production, purification and characterization of potent antibacterial compound
   3.4.1 Extraction of antibacterial produced by actinomycetes
   3.4.2 Effect of pH on antibacterial activity
   3.4.3 Effect of temperature on antibacterial activity
   3.4.4 Extraction of antibacterial by different solvents
   3.4.5 Purification by column chromatography
   3.4.6 Physicochemical and biological properties of antibacterials
      3.4.6.1 Physical properties of antibacterials
      3.4. 6.2 Biological properties of antibacterials
      3. 4. 6.3 Chemical properties of antibacterial JH1MN135857 and CB2MN13585
      3.4.6.3.1 Antibacterial JH1MN135857
      3.4.6.3.2 Antibacterial CB2 MN135853.

CHAPTER FOUR: SUMMARY AND CONCLUSION
CHAPTER FIVE : REFERENCES
PUBLICATIONS

CHAPTER ONE

INTRODUCTION

1. Introduction:

Hadhramout governorate lies in the eastern part of Yemen. It is comprised of different topography distributed between the coastal plains containing beautiful shores of the Arabian sea, mountains and hills of height reaching 2000 meters above the sea level. In the hot tropical climate of Hadhramout, the temperature reaches more than 40ºC in summer interior areas, where continental dry climate prevails. The temperature reaches 36ºC in the coastal areas, due to seasonal winds saturated with moisture and the temperature in winter tends to be moderate in the coastal areas with 20-24ºC and 17-20ºC in the interior areas.

The main aim is sampling in caves, mountains and dams for this research, since this is the first study in the Middle East to isolate actinomycetes from this special habitat in Hadhramout Yemen.

1.1 Actinomycetes

The presence of actinomycetes has been known for over hundred years. For much of the time they were seen as a foreign group of organisms with an attraction to both bacteria and fungi. However, the study of their fine structure and chemical composition started in the 1950s, confirmed their prokaryotic nature. They now create the order Actinomycetales and their removal from the mycologists, effective reach has been finished. Their difference in status paralleled that of the blue-green growth to the cyanobacteria but it was accepted more rapidly and less acrimoniously. It is not easy to give a short, accurate definition of actinomycetes. They are frequently described as bacteria, which have the ability to form branching hyphae at some stage of their development. However, this attribute is tenuous, and it often requires the imagination to believe in it (Gottlieb, 1973). The name "Actinomycetes" was derived from Greek "atkis" (a ray) and "mykes" (fungus) and has features of both Bacteria and fungi (Das *et al.,* 2008). Actinomycetes are soil organisms, which have features common to bacteria and fungi but the possess sufficient distinctive features to delimit them into a distinct group. They were initially erroneously classified as fungi since they have true aerial hyphae and form spores, both of which are thought to be fungal characteristics (Lechevalier and Lechevalier, 1967).In general, actinomycetes are Gram-positive, but part of their thallus may be gram negative. Certain filamentous, branching Gram-negative bacteria. Certain filamentous, branching, Gram-negative bacteria belonging to the genus mycoplana may be classified with actinomycetes as a matter of convenience, although they are not phylogenetically related to other members of the order (Sukapure *et al.,* 1970) In general, actinomycetes will grow on ordinary laboratory media for example, nutrient agar, trypticase soy agar, blood agar, and even brain-heart infusion agar, but their growth is usually slower than that of ordinary

bacteria. A division cycle in actinomycetes may take 2-3 h as compared with 20 min for Escherichia coli. Some actinomycetes grow even more slowly. Mycobacterium tuberculosis, for example, has generation time of about 15 h under optimal conditions. Other actinomycetes, such as M. leprae, have never grown on laboratory media. Some endophytes of plants placed in genus Frankia; grow on present-day laboratory media but with greatest difficulty (Lechevalier and Lechevalier, 1967). Actinomycetes show outgrowths from a spore or fragments of mycelium (Colony-forming units, CFUs) develop into hyphae that penetrate the agar (substrate mycelium) and the hyphae that branch repeatedly become fixed on the surface of the agar to form a strong colony. The density and consistency of the colony will depend on the composition of the medium. Nocardioform actinomycetes exhibit fragmentation; the hyphae breaks up into rods and cocci, thus leading to soft or friable colonies. In strains of certain genera (e.g. Streptomyces), the colony becomes covered with aerial mycelium: free, erect hyphae surrounded by a hydrophobic sheath that grow into the air away from the colony. The surface of transparent agar can be seen with the aid of a microscope after 24 hours, and visible colonies may appear in 3-4 days, but mature aerial mycelium with spores may take 7-14 days to develop, and some very slow growing strains may require up to 1 month's incubation. The saprophytic actinomycetes are oxidative and may grow poorly when the air supply is restricted. Actinomycetes can grow in broth but need to be cultivated under specialized conditions. The growth of Streptomycetes in a stationary broth tube is usually restricted to a surface pellicle and perhaps a cottony sediment, leaving the broth quite clear. Liquid cultures require considerable aeration and agitation to give the suspended growth. Tubes and flasks must be incubated on a shaker at high speeds (e.g. 200 – 250 rpm) to give the supply of oxygen and mixing necessary for maximum growth. Even, diffuse mycelial growth may require the higher agitation and mixing rates achieved by baffles or springs (Goodfellow and Williams, 1983). Actinomycetes are gram-positive bacteria belonging to the order Actinomycetales (Lechevalier, 1981), characterized by having a high Guanine and Cytosine content in their Deoxyribonucleic Acid. The composition of the growth media can profoundly affect the growth and stability of substrate and aerial mycelium (Kalakoutskii and Agre, 1976). The majority of actinomycetes are soil bacteria that play an important role in mineralization processes in nature. Actinomycetes form a very important class of bacteria since they produce numerous natural products such as antibiotics and enzymes (Edwards, 1993).Actinomycetes have possessed many significant and interesting features. They have given many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been uncommonly successful, and around

2/3 of naturally happening antibiotics, including many of medical significance, have been isolated from actinomycetes (Okami and Hotta, 1988). Secondary metabolites are synthesized from primary metabolite precursors. Studies of primary metabolism in these types of organisms have been very limited but would provide important information about the switch between primary and secondary metabolism and for further improvement of industrial processes for the production of secondary metabolites.

One consequence of intense screening programs carried out over the past several years is that there is a developing issue of "rediscovery" already known bioactive compounds (Nolan and Cross, 1988). This trend appears to suggest that the easily accessible actinomycetes in the soil had been depleting and there is a need to look for unutilized microorganisms from unexplored sources (Igarashi, 2004). One way to approach this issue is to extend the source of actinomycetes via ecological evaluations of environments other than earthly soils. There is also growing interest in the non-Streptomycetes actinomycetes as sources of novel compounds (Nolan and Cross, 1988). It is likely that the assorted variety of secondary metabolites depend pretty much on the isolation source, viz the environment of the producers (Igarashi, 2004). Based on above certainties new actinomycete strains that create active compounds have been as of late isolated from novel sources including saline, sea, mangrove woodlands and specialty natural surroundings, for example, caves, beehives, pristine forests, lakes, rivers and other wetlands (Radhakrishnan, *et al*., 2010) to cope with the demand for new pharmaceutical compounds and to resist the antibiotic-resistant pathogens.

1.2 Scope:

Research on novel actinomycetes constitutes an essential component in natural product-based drug discovery. Pharmaceutical companies screen thousands of *Streptomyces* strains annually as a potential source of novel chemical compounds but it is becoming increasingly difficult to obtain novel compounds, and screening usually results in the re-isolation of known compounds (Watve *et al.,* 2001). Metagenomic and mathematical studies have, however, shown that the antibiotic producing organisms have a large potential to produce more compounds (Watve *et al.,* 2001; Kennedy *et al.,* 2007). The study of actinomycete strains from mountain, dam and cave are important because they are an extreme environment, which have not been extensively studied but is believed to have a large diversity of organisms that have not yet been discovered. On this basis, it is presumed that the chance of isolating novel actinomycetes from these types of soils is high.

2. Aim and Objectives

2.1 Aim

Isolation and identification of antibacterial activity from actinomycetes isolated from some special soil habitats of Hadhramout–Yemen.

2.2 Objectives:

1. Isolation of actinomycetes from some dams, caves and mountain soils of Hadhramout –Yemen.
2. Screening of actinomycetes for antibacterial and enzymatic activity.
3. Identification of actinomycetes, which have strong antibacterial activity.
4. Study on the production, purification and characterization of potent antibacterial compound.

CHAPTER TWO

LITERATURE REVIEW

2. Literature review

2.1 Non-Streptomyces from special habitats:

2.1.1 Caves as special habitats

Caves are generally nutrient-poor habitats with low temperature and high humidity (Barton, 2006). They are witnessed as excellent sources for the discovery of novel strains of actinomycetes (Ara *et al*., 2008). Several new species have been isolated from cave soils including *Catelliglobosispora koreensis* from a gold mine cave in Korea (Jurado *et al*., 2005a), *Agromyces salentinus* and *Agromyces neolithicus* from the Grotta Dei Cervi cave in Italy (Jurado *et al*., 2005b)and *Agromyces subbeticus* from a cave occupied by bats in Spain (Lee,2006a). Recently, *Amycolatopsis jejuensis, Amycolatopsis halotolerans, Nocardia jejuensis* and *Nocardia speluncae* have been isolated from natural caves (Lee, 2006b, Soe *et al*., 2007 and Laiz *et al*., 1999). Caves are one of the habitats inviting microbiologists' attention during the past decade (Groth *et al*., 1999,) with a combination of unique conditions including high humidity, wetness, relative low and stable temperature (Laiz *et al*., 2003). Many microbiologists believe that microbes collected from original sites that were unknown or infrequently visited by humans were likely to be novel taxa or strains, which produce unique useful chemical compounds. In an early work, great variety of cultivable actinomycetes was found in the Altamira cave, Cantabria, Spain (Groth *et al*., 1999 and Laiz *et al*., 2003), Tito Bustillo, Spain (Laiz *et al*., 2003, Groth *et al*., 2001 and Jurado *et al*., 2008) and Grotta dei Cervi, Italy (Jurado *et al*., 2008). Many novel species have been discovered from cave environments, such as *Actinocorallia cavernae* (Jurado *et al*., 2008), *Agromyces subbeticus* (Lee, 2006a), *Amycolatopsis halotolerans* (Soe *et al*., 2007) *Nocardia altamirensis* (Groth *et al*., 2002), *Nocardia speluncae* (Laiz *et al*., 1999) including novel genera such as *Knoellia* (Groth *et al*., 2002) and *Hoyosella* (Nakaew *et al*., 2009a). According to a recent review by Tiwari and Gupta (2012), this non-Streptomycetes actinomycetes group comprising Actinomadura, *Actinoplanes, Gordonia, Microbispora, Micromonospora, Nocardia, Nonomuraea* and *Saccharopolyspora*, were isolated from tropical limestone caverns at Khao No-Kh Kaeo karst. Moreover, Niyomvong *et al*., (2012), they were isolated 8 different genera namely *Actinomadura, Actinoplanes, Gordonia, Microbispora, Micromonospora, Nocardia, Nonomuraea* and *Saccharopolyspora* from tropical limestone caverns at Khao No-Khao Kaeo karst, Thailand, Thailand caves Successful isolation of members of the genus *Actinomadura* and *Saccharopolyspora* from the caves was reported for the first time. The list has further been extended by reports much attention has been given to isolate none Streptomyces from the caves as well as uncommon extreme environments.

Numerous rare actinomycetes have been isolated from variety caves of Europe, such as Altamira (Jurado *et al*., 2005a, b), Tito Bustillo and Llonn caves in Spain (Soe *et al*., 2007, Laiz *et al*., 1999, 2003 and Groth *et al*., 1999, 2001) and Grotta dei Cervi, Italy (Jurado *et al*., 2008) resulted in the isolation of rare genera such as *Amycolatopsis, Microbacterium, Nocardia* and *Rhodococcus*. In Asia, there were only reports from China, Korea and Thailand. A study by Nakaew *et al.*, (2009b) reported the isolation of several rare actinomycete genera (*Actinocorallia, Catellatospora, Microbispora, Micromonospora, Nonomuraea, Pseudonocardia, Saccharothrix* and *Spirillospora*) from Phatup Cave Forest Park and Phanangkhoi cave in northern Thailand. These findings strongly suggested that non-Streptomyces are an indigenous population of microbial community inhabited in caves.

2.1.2 Dams as special habitats:

Waksman (1959) clarified that actinomycetes were rich in freshwater lakes. An old report expressed that thermophilic actinomycetes were found in river water. They were additionally found in sewage and developed well at 60°C. This statement was then proved when Cross (1981) reported the isolation of individuals from genera *Actinoplanes*, *Micromonospora*, *Rhodococcus*, Streptomyces and the endospore-framing *Thermoactinomycetes* from freshwater habitates (Goodfellow Haynes, 1984).
However, he explained that the majority of these actinomycetes most probably were washed-in from land and accumulated in freshwater habitats. According to Makkar and Cross (1982), sporangia of *Actinoplanes* could withstand lengthily dryness and release motile spores when rehydrated. That is the reason they are common in soils, dams, and lakes. *Actinoplanes* are mainly discovery on *Allochthonous* leaf litter washed to the lakeshore and twigs submerged in streams. Waksman believed that individuals from the genus *Micromonospora* represented a truly indigenous group of microbial occupants of the waters and base stores of inland lakes (Waksman, 1959). Therefore, a great number of *Micromonospora* also can be isolated from lake sediments. Complex organic compounds, such as, chitin, cellulose, and lignin accumulated in lake mud were decomposed by the genus of actinomycetes. Rowbotham and Cross (1977) explained that *Micromonospora* spores can survive as inactive propagules as they washed into streams, rivers, and lakes. Comparable to *Thermoactinomycetes endospores* found in lake sediments, it was also reported that they remain inactive in cool waters as they were washed in from surrounding soils. On the other hand, the present of *Rhodococcus coprophilus* (also has been isolated from water and sediments of rivers and lakes); a coprophilic species in lakes was believed due to washing in of contaminated herbivore dung. Study of freshwater

actinomycetes isolation from sediments of lakes was carried out by Xu *et al*., (1996) resulted the discovery of yielding mainly *Micromonospora* followed by Streptomyces. The presence of *Streptomyces* in the freshwater habitat was because of their spores being always washed into the rivers and lakes. This allows the Streptomyces spores to be found as foam of waterfalls at the water-air interface.

2.13 Mountains as special habitats:-

Many studies have been conducted in the search for novel actinomycetes isolated from a mountain for production of new antibiotics. From these studies are Xu *et al*., (1996) studied the distribution of actinomycetes in soils of Yunnan, China, and announced that their genetic assorted variety was more intricate in subtropical and tropical zones than in cool-mild mountain and snow-mountain zones. According to a recent review by Andayani *et al*., (2015), the few species have been isolated from Tangkuban Perahu mountain soil including *Nocardia sp YIM 65630*, *Streptomyces galbus* and *Nocardia niigatensis*. Respectively. Production of antibacterial, antifungal and anticancer metabolites. The list has further been extended by recent reports of non-Streptomyces as rare genera isolated from mountains; *Actinomadura meridiana sp. nov*. actinomycetes were acquired soil from the surface of a stone gathered from the pinnacle of Darangshi Oreum (Small Mountain) in Jeju, Republic of Korea(Lee ,2012), *Nonomuraea soli sp*. nov. isolated from Weibao Mountain in south-west China(Cao *et al*., 2012), *Nocardia niigatensis NR 043916.1 isolated from soil of Tangkuban Perahu mountain*, Indonesia (Sukandar *et al*.,2014), *Streptosporangium spp*. isolated from soil Taibai Mountain, Shaanxi Province, China (Wang *et al*., 2013). *Micromonospora marina; Micromonospora aurantiaca; Micromonospora chalcea; Micromonospora chokoriensis; Micromonospora tulbaghiae and Micromonospora chersina* were isolated from were isolated from mountain soils in Phukradung, Loei province, the North Eastern part of Thailand (Songsumanu *et al*., 2013).

CHAPTER THREE

RESULTS

3. Results

3.1 Isolation of actinomycetes from some dams, caves and mountain soils of Hadhramout – Yemen.

3.1.1 Physiochemical properties of soil sample and isolation of actinomycetes

Actinomycetes are common in terrestrial habitats such as dams, caves, mountains, agriculture fields and forest soils where they form a major constituent of the actinomycete community. In contrast, members of this group grow within pH range 6.5 to 10.0 with an optimal around 7.0. As well as, temperature from 20℃ – 45℃ with an optimal around 37℃ (Fig.1)

Soils from different regions of three districts viz., Sah, Ghayl Bin Yamin and Ash-Shihr about 19 soil samples from 19 locations in three districts were analysed over a period of six months for physicochemical parameters and actinomycetes population. The physicochemical analysis included temperature, pH, and nature of the soil samples. Isolation and enumeration of actinomycetes performed by soil dilution plate technique using Starch Casein Agar (SCA) of pH 7.0, incubated for 7 days at 37 ºC. The isolated colonies were maintained on starch casein agar slants.

In the present study, 219 actinobacteria colonies from 19 different location of the terrestrial and aquatic ecosystem were isolated. Among the 219 colonies, 103 isolates were morphologically different. The maximum isolates were isolated in dam soils followed by cave, mountain soils and few of them agriculture fields (Fig. 3).

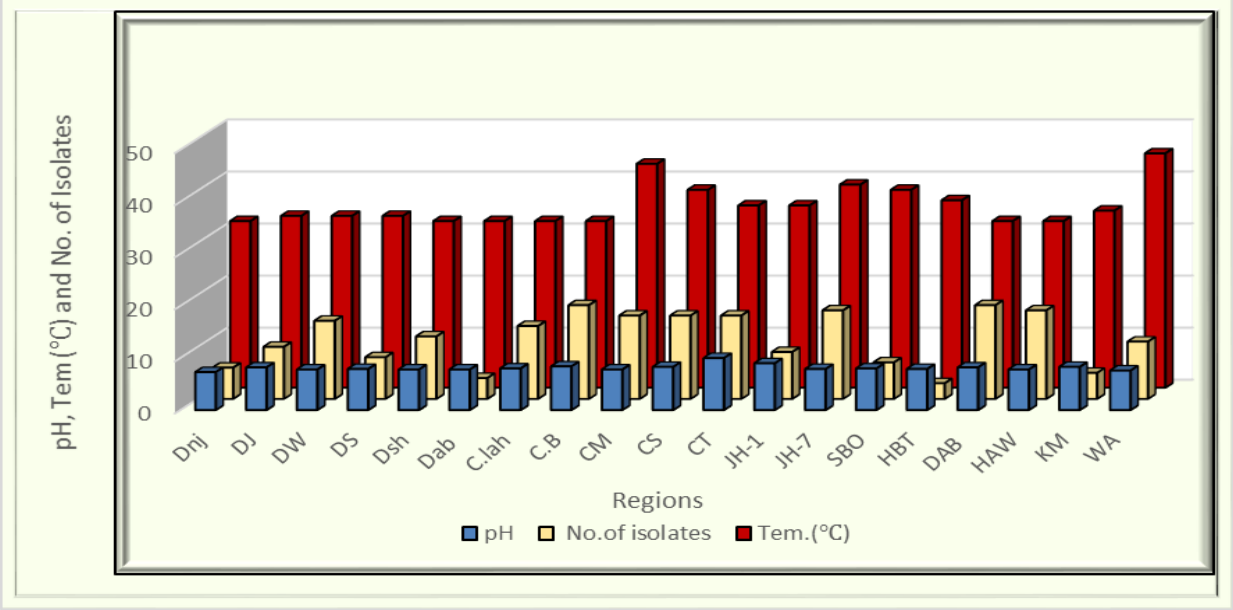


Fig. 1: Frequency of occurrence of actinomycetes and their pH and temperature**.**

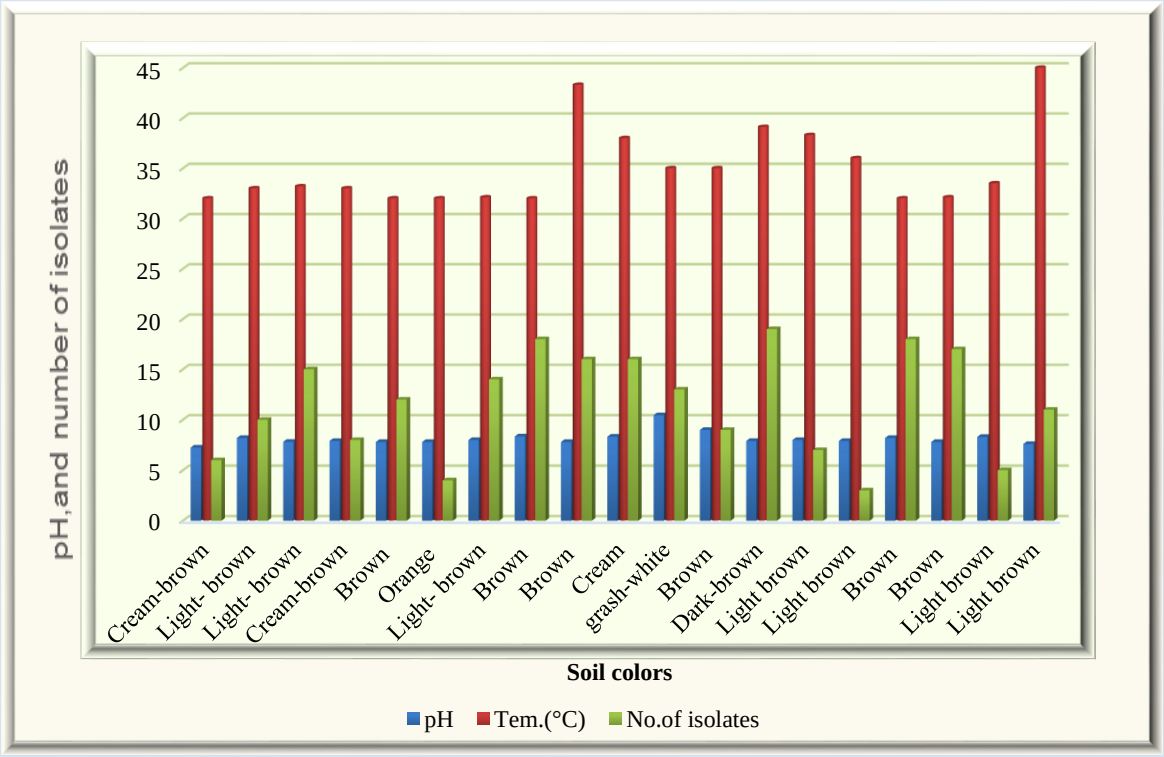


Fig. 2: Soil-color frequency of occurrence of actinomycetes and their pH and temperature.

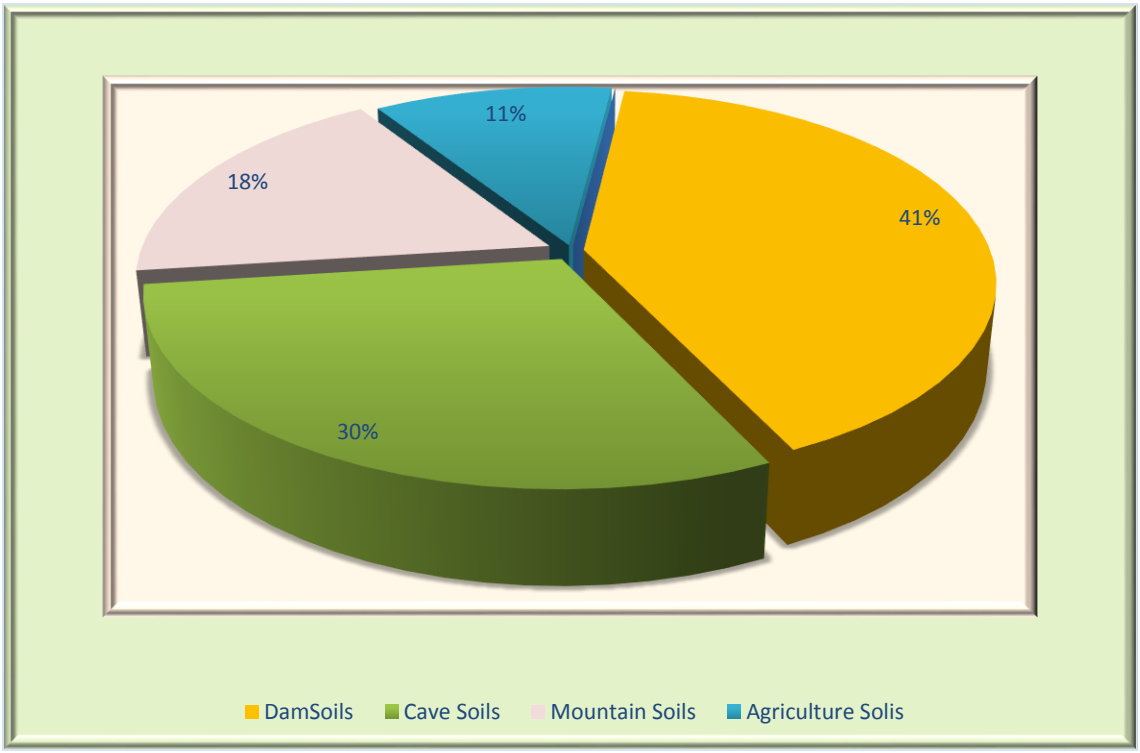


Fig. 3: Site- wise distribution of actinomycetes.

Soil samples were collected from dams, caves, mountains, and agricultural fields. pH, temperature and soil color, of all soil samples, varied. The present study clearly indicated that physicochemical parameters have an influence on the biodiversity and distribution of actinobacteria in Ghayl Bin Yamin district, Sah district and Ash-Shihr district.

3.2 Screening of actinomycetes for antibacterial and enzyme activities:

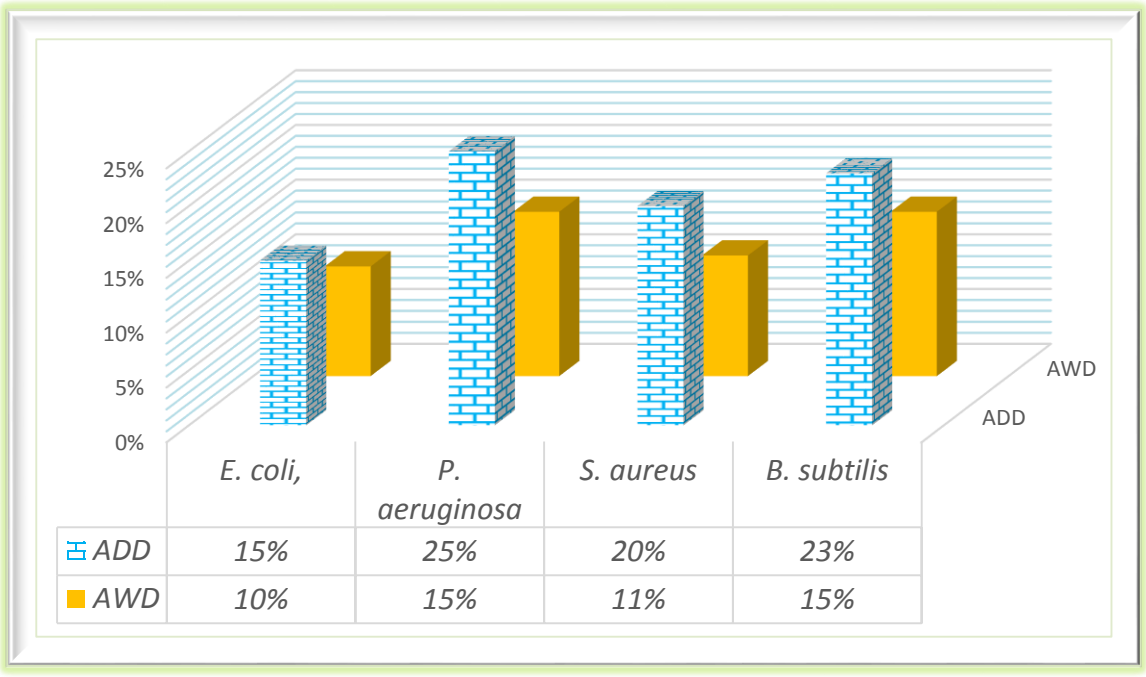
3.2.1 Selection of actinomycete from different soils:

From 219 actinomycete isolates, 103 isolates (47%) selected for study according to color and their activity against bacterial pathogens.

3.2.2 Screening of actinomycete isolates for antibacterial activity:

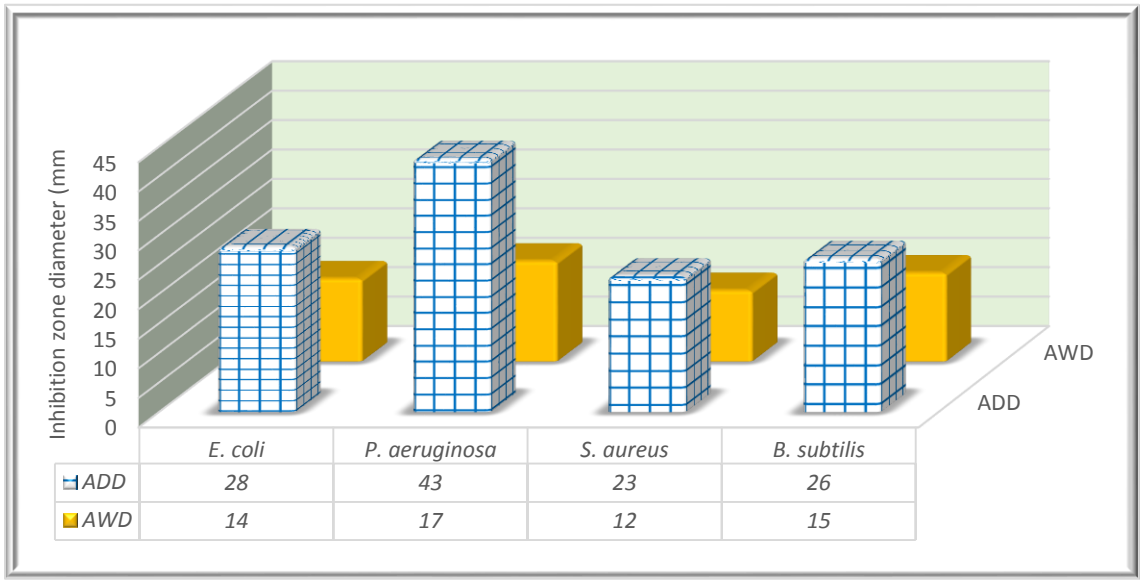
All the isolates screened for antibacterial activity, by agar disc diffusion method (ADD) and by agar well diffusion method (AWD). Antibacterial activity was studied against Gram-negative and Gram-positive bacteria. The target cultures used were *E. coli, P. aeruginosa, S. aureus*, and *B. subtilis*. Out of 103 actinomycetes isolates, there were 24 isolates (23%) shown activity against B. subtilis by ADD and 15 isolates (15%) by AWD. For *S. aureus* 21 isolates (20. %) had activity by ADD and 11 isolates (11%) had activity by AWD. There were 26 isolates (25%) showing activity against *P. aeruginosa* by ADD and 15 isolates (15%) by AWD. The activity against *E. coli* was very low, only 16 isolates (15%) had shown activity by ADD and 10isolates (10%) had shown activity by AWD as shown in Fig. 4

The ADD method that used for screening antibacterial was more effective and gave better results than AWD as shown in Fig. 5. For *B. subtilis* the maximum inhibition zone diameter was 26mm. by ADD technique but by AWD the zone was less than 15mm. for *S. aureus* the maximum inhibition zone was 23mm. by ADD technique and 12mm.by AWD. For *E. coli* the maximum inhibition zone diameter was 28 mm. by ADD technique, but was 14 mm by AWD. The inhibition zone of *P. aeruginosa* was 43 mm by ADD, but was 17 mm by AWD (10).



	E. coli,	P. aeruginosa	S. aureus	B. subtilis
ADD	15%	25%	20%	23%
AWD	10%	15%	11%	15%

Fig. 4: Percentage of antibacterial activity of actinomycete isolates.



	E. coli	P. aeruginosa	S. aureus	B. subtilis
ADD	28	43	23	26
AWD	14	17	12	15

Fig. 5: Maximum inhibition zone of antibacterial activity of actinomycete isolates by (ADD) and (AWD).

3.2.3 Antibacterial activity on different media:

In all 26 actinomycetes, isolates were grown on five different media such as SCA, SYA, GAA, GYA, and NA, furthermore, tested for their antibacterial activity. SCA stimulated maximum antibacterial activity amongst all media. The inhibition zone diameter ranged from 16 mm to 23 mm against *P. aeruginosa*. The GAA medium was second and GYA third in stimulating antibacterial activity. Least stimulation of antibacterial activity was on NA medium (Fig. 6).

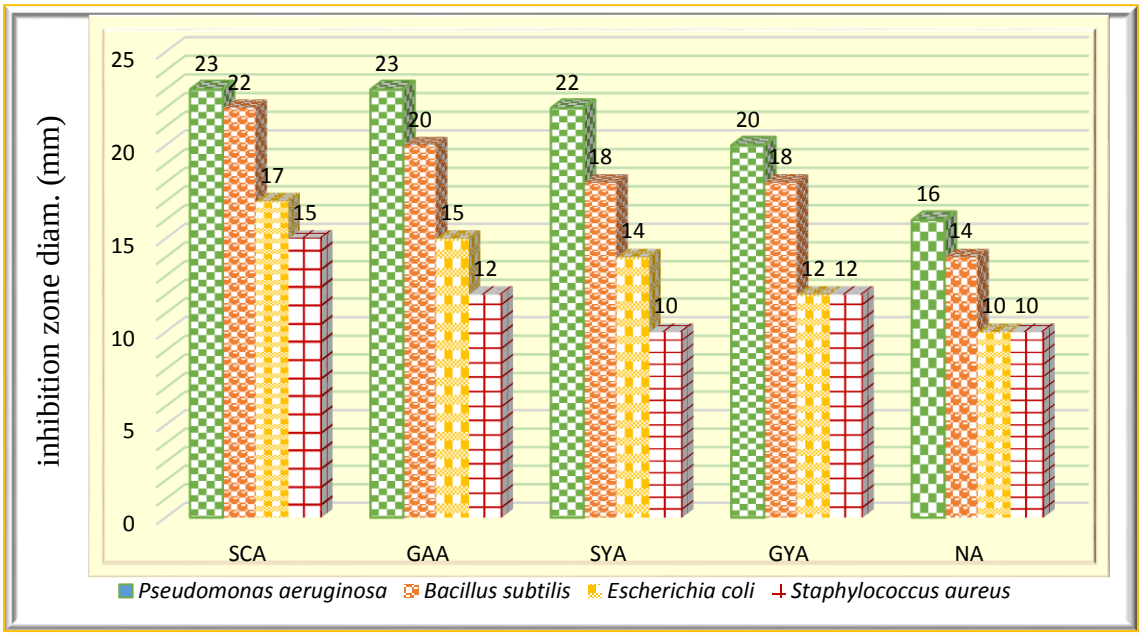


Fig. 6: Antibacterial activity of actinomycete grown on different media.

3.2.4 Enzyme Activities:

All the selected isolates included strictly actinomycetes, which have strong antibacterial activities, were screened for following enzyme activities (Fig.7).

- **Amylase Activity:**

All the isolates screened for amylase activity on starch agar exhibited good amylase activity. Plates were flooded with iodine solution; colorless zones of starch hydrolysis were observed around the colonies against the blue background. Out of all the isolates, 81% (21) produced amylase.

❖ **Chitinase Activity:**

For detection of chitinase activity, all the isolates were grown on colloidal chitin. Zones of chitin hydrolysis were observed within 8 days of incubation. On further incubation, zones of chitin hydrolysis increased in size. Zone of chitin hydrolysis was recorded. Out of all the isolates, 77% (20) produced chitinase.

❖ **Casein hydrolysis Activity:**

All isolates were screened for casein hydrolysis activity on casein agar (CA). In all, 81% (21) isolates produced caseinase.

❖ **Pectinase Activity:**

Pectin agar (PA) was used for screening of pectinolytic activity of all the isolates. To observe pectinase activity the plates with growth were flooded with cetrimide for 10 minutes. Clear zones around colonies indicated pectinolytic activity. Of the total isolates, 73% (19) produced pectinase.

❖ **Gelatinase Activity:**

All the isolates were screened for gelatinase activity on gelatin agar (GA), 77% (20) of the isolates exhibited gelatinase activity.

❖ **Keratinase Activity:**

All actinomycete isolates were screened for keratinase activity on keratin agar (KA) with pulverized chicken feathers. Of the total isolates tested, 73% (19) produced keratinase.

❖ **Lecithinase Activity:**

All isolates were screened for lecithinase activity on egg yolk agar (EYA). Of the isolates tested, 81% (21) exhibited lecithinase activity.

❖ **Lipase Activity:**

All actinomycete isolates were screened for lipolytic activity on tributyrin agar (TA) plates. Only 42% (11) of the total isolates were lipase positive.

❖ **Urease Activity:**

Only 92% (24) of actinomycete isolates were producing urease in urea agar (UA) plates.

❖ **Catalase activity:**

All actinomycete isolates were screened for presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase. Only 70% (18) of the total isolates were catalase positive .

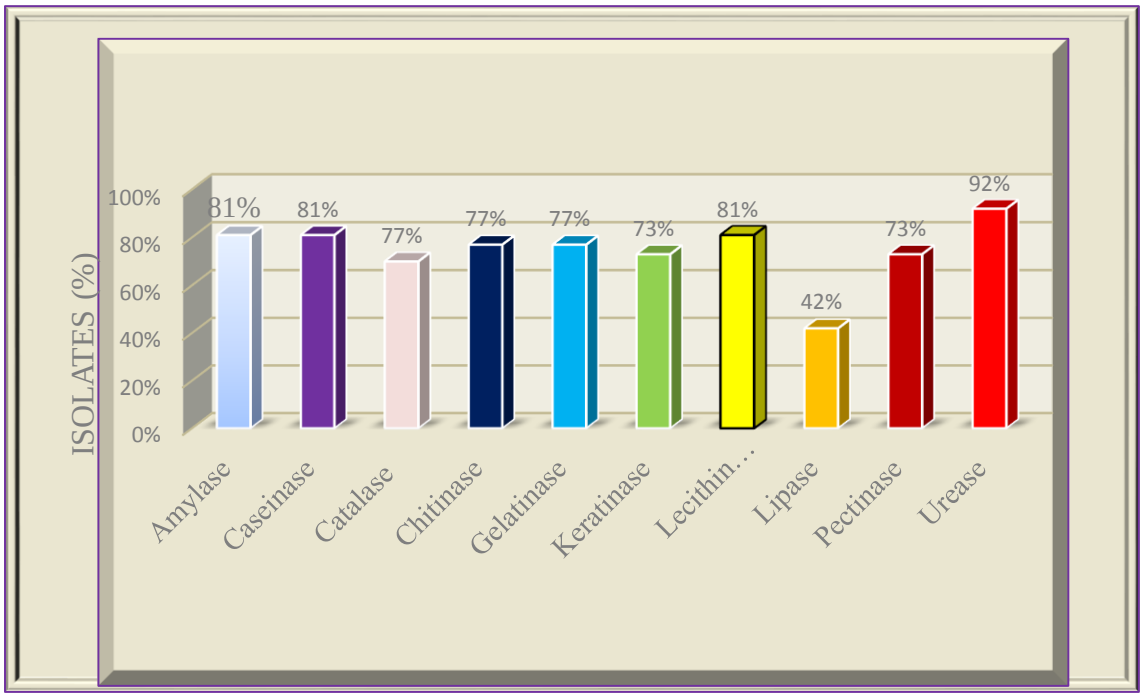


Fig. 7: Percentage of enzyme activity of actinomycetes isolates

This investigation showed that all of the isolates varied between the positive and negative results of enzymatic activity such as caseinase, amylase, lipase, lecithinase, keratinase, chitinase, and gelatinase. From the results, it is clear that the ability of actinomycetes to produce a huge number of enzymes indicates the diversity of the group of actinomycetes and has many applications, which has increased the importance of this group of actinomycetes.

3.3 Identification of actinomycetes, which have strong antibacterial activity

In all, twenty-six actinomycete isolates were identified as belonging to three genera as shown in table 1. Out of them, twenty accurately belonging to *Streptomyces* and six belong to other genera. Out of them, four isolates belonging to *Nocardia* and two isolates were identified as *Actinoplane.* The taxonomic position of all strain isolates was established based on 16s rRNA analysis. The sequence of 16s rRNA was compared with sequences in NCBI Blast. Evolutionary trees were constructed based on the maximum method of analysis using the boot analysis and were deposited in the gene bank with the accession number. In addition to morphological, cultural, physiological, biochemical and molecular analysis, all strains were determined to the strain level.

Our results demonstrated that the isolates belonged to Streptomycetes were foremost in all sites. Isolates belonged to *Streptomyces*, *Nocardia* and *Actinoplanes*.

Streptomyces:

There were 77 % of the isolates, which belonged to these genera. Substrate mycelium was none-fragmenting and branched. Arial mycelium formed short or long spore chains. Spore chains were straight, hooked or spirals. On starch casein agar, glycerol- asparagine agar, glycerol- yeast extract agar and starch yeast extract agar, the growth was good and fast. They produced a wide variety of diffusion and melanin pigment. The isolates were growing within the mesophilic.

ii) *Actinoplanes*:

There were 7.69 % of isolates, which suited to this genera. Substrate mycelium was fine and branched. Aerial mycelium was scanty in all isolates.

iii) *Nocardia*:

There were 15.38% of isolates belonging to nocardioform group. Substrate mycelium was branched and fragmented in to bacillary elements. Most of genera do not have aerial mycelium and other genera have being sparse and invisible aerial mycelium. *Nocardia* genus was acid-fast bacteria.

Table 1: Taxonomy of actinomycetes.

Isolate no.	Total isolate	Identified of strains isolate	Accession number
Streptomycetes sp			
C.B-2	3	***Streptomyces thermocarboxydus CB2***	**MN135853**
C.S-3	3	***Streptomyces gobitricini CS3***	**MN135852**
C.T-2	1	***Streptomyces lilaceus CT2***	**MN135854**
DJ-1	1	***Streptomyces variabilis DJ1***	**MN1357855**
DW-3	2	***Streptomyces rochei DW3***	**MN135854**
DAB-11	1	***Streptomyces rochei DAB11***	**MN567711**
JH-1	1	***Streptomyces levis JH1***	**MN135857**
Dab3	2	***Streptomycetes luridiscabiei Dab3***	**MN559792**
Dsh-2	1	***Streptomyces carpaticus Dsh-2***	**MN559789**
Hbt-3	1	***Streptomyces enissocaesilis HBT3***	**MN559788**
SBO-3	1	***Streptomyces plicatus SBO3***	**MN559790**
DJ-3	1	***Streptomyces alfalfae DJ3***	**MN559791**
CS-2	1	***Streptomyces sp CS2***	**MN567712**
CM-2	1	***Streptomyces sp CM2***	**MN559793**
Nocardia sp			
DS-2	1	***Nocardia kroppenstedtii DS2***	**MN567716**
C.Lah-3	2	***Nocardia asteroids C.Lah3***	**MN567714**
JH-1	1	***Nocardia otitidiscaviarum JH7***	**MN567715**
Actinoplane sp			
Dnj-2	2	***Actinoplanes digitatis Dnj2***	**MN567713**

3.4 Study on the production, purification and characterization of potent antibacterial compound

3.4.1 Extraction of antibacterial produced by actinomycetes:

Four actinomycete isolates were selected for fermentation. Starch casein broth pH 7.0 was used and incubated at 37℃ for 21 days. The selected isolates from different genera were identified as *Streptomyces levis* JH1 MN135857, *Streptomyces thermocarboxydus* CB2 MN135853, *Nocardia kroppenstedtii* DS2MN567716, and *Actinoplane digitatis* Dnj2MN567713.

The diversity of actinomycetes isolated was behind the selection of these isolates for further study. The production of different pigments, antibiotics, enzymes, and other properties was giving this group-wide diversity.

The growth of four actinomycete isolates on SCB media to produce antibacterial was carried out in a 300ml flask. The color of growth media was observed in plate no.1. The isolate *Streptomyces levis* JH1 MN135857, which produced antibacterial antibiotic and produce light yellow pigment. This color was antibacterial. This antibacterial gave a maximum zone of inhibition for *B. subtilis, S. aureus, E. coli,* and *P. aeruginosa.*

The growth carve of *Streptomyces* genera (*Streptomyces levis* JH1 MN135857, *Streptomyces thermocarboxydus* CB2 MN135853 were recorded, started for produce antibacterial after 3 days of incubation and maximum of antibiotics production after 8th days of incubation.. On the other hand, the growth curve of the *Nocardia kroppenstedtii* DS2MN567716, and *Actinoplane digitatis* Dnj2MN567713 were recorded, started to produce antibiotics after 7 days of incubation and maximum of antibacterial production between 14-21days of incubation.

Most of *Streptomyces* species grew faster than other genera and reach to stationary stage,

Streptomyces levis JH1 MN135857and *Streptomyces thermocarboxydus* CB2 MN135853 grew faster than other genera and reach stationary sage on the 8th day of incubation at OD 0.580 and 0.600 at A540 respectively. As well as, the maximum growth OD 0.482 at A540 for isolate *Nocardia kroppenstedtii* DS2MN567716 and minimum growth OD 0.342 at A540 for isolate *Actinoplane digitatis* Dnj2MN567713 (Fig. 51).

The maximum zone of inhibition produced by *Streptomyces levis* JH1 MN135857 against *B. subtilis* (3.5 cm), *S. aureus* (2.6 cm), *E. coli* (1.8 cm) and *P. aeruginosa* (2cm)) and the maximum zone of inhibition produced by *Streptomyces thermocarboxydus* CB2 MN135853 against *B. subtilis* (3.4 cm), *S. aureus* (2.4 cm), *E. coli* (1.4 cm) and *P. aeruginosa* (2.2 cm) as well as the maximum zone of

inhibition produced by *Nocardia kroppenstedtii* DS2MN567716, against *B. subtilis* (2.3 cm), *S. aureus* (1.6 cm), E. coli (1.4 cm) and *P. aeruginosa* (2.8 cm). On the other hand, the maximum zone of inhibition produced by *Actinoplane digitatis* Dnj2MN567713 against *B. subtilis* (2.4 cm), *S. aureus* (1.5), *E. coli* (1.3 cm) and *P. aeruginosa* (2.2 cm).

The four isolates from fermentation broth was incubated for 21 days, then extracted the crude antibiotics by ethyl acetate 1:1(V/V)and stored in Eppendorf tubes at 4℃ .

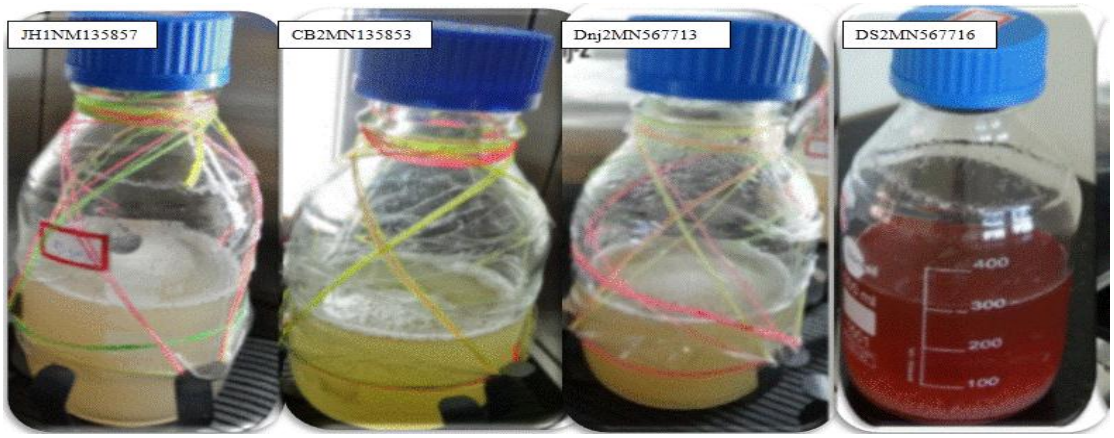


Plate 1: Fermentation of actinomycetes culture on SCB.

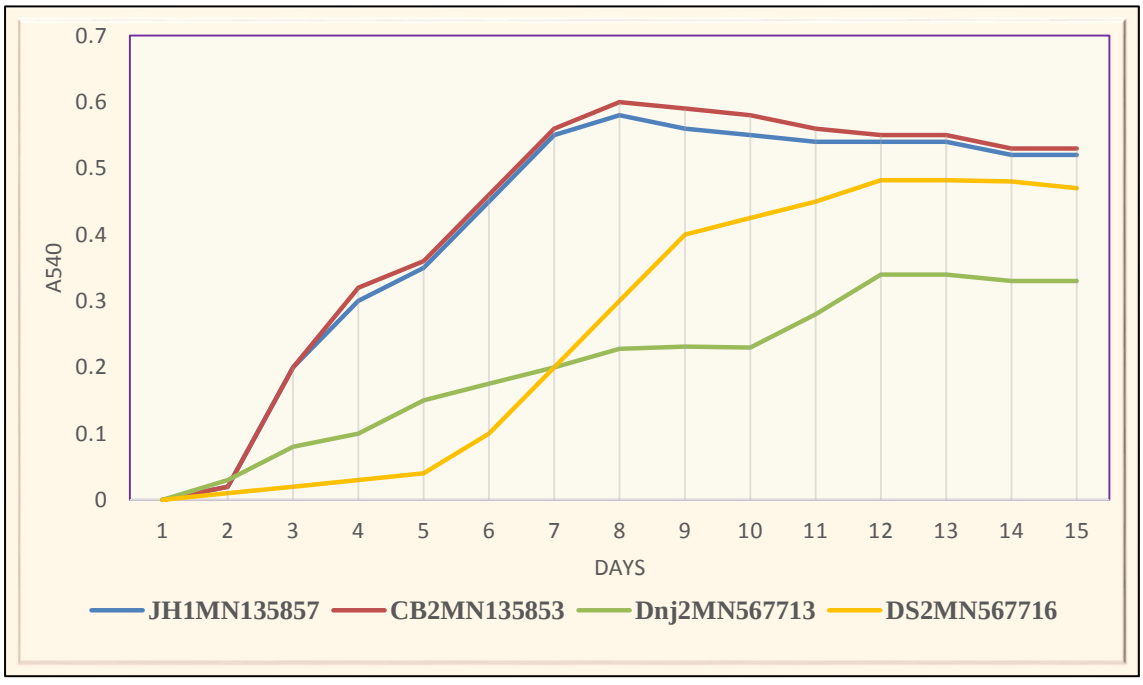


Fig 7: Growth curve of selective actinomycetes isolates.

3.4.2 Effect of pH on antibacterial activity:

These selected isolates showed activity against *B. subtilis S. aureus E. coli* and *P. aeruginosa*. All the crude antibiotics are active and stable against *B. subtilis, S. aureus, and E. coli and P. aeruginosa at* different pH. All the crude antibiotics were active at different pH but maximum activity at pH 7 for all antibiotics. There were no much differences in the activity of antibacterial antibiotics for different bacterial strains. Antibacterial activity of most isolates gave a maximum zone of inhibition for *B. subtilis.*

Table 2 Effect of different pH on crude antibacterial activity against *B. subtilis, S. aureus,* and *E. coli and P. aeruginosa in* NA media (zone of inhibition in cm).

Anti. No.	***B. subtilis***			***S. aureus***			***E. coli***			***P. aeruginosa***		
	4.5	**7.0**	**10.0**	**4.5**	**7.0**	**10.0**	**4.5**	**7.0**	**10.0**	**4.5**	**7.0**	**10.0**
JH1MN135857	1.8	**3.5**	2.0	1.5	**2.6**	2.6	1.2	**1.8**	0.9	1.8	**2.0**	1.2
CB2MN135853	1.4	**3.4**	1.8	1.2	**2.4**	2.0	1.0	**1.4**	0.8	1.6	**2.2**	1.0
Dnj2MN567713	2.0	**2.4**	1.5	1.0	**1.5**	1.0	1.2	**1.3**	1.0	2.0	**2.2**	1.0
DS2MN567716	2.2	**2.3**	1.3	1.6	**1.6**	1.3	1.2	**1.4**	1.0	2.4	**2.8**	1.6

4.4.3 Effect of temperature on antibacterial activity:

These four isolates showed activity against *Bacillus subtilis, S. aureus, and E. coli and P. aeruginosa* Most of the crude antibiotics were active and stable against *B. subtilis* at different temperatures except 45℃ and maximum activity was at 37℃.

Table 3: Effect of different temperature on crude antibacterial activity *B.subtilis, S. aureus, and E. coli* and *P. aeruginosa* in NA media (zone of inhibition in cm).

Anti. No.	***B. subtilis***			***S. aureus***			***E. coli***			***P. aeruginosa***		
	28℃	**37℃**	**45℃**	**28℃**	**37℃**	**45℃**	**28℃**	**37℃**	**45℃**	**28℃**	**37℃**	**45℃**
JH1MN135857	3.2	**3.5**	-	2.1	**2.6**	-	1.8	**1.8**	-	2.0	**2.0**	-
CB2MN135853	3.	**3.4**	-	2	**2.4**	-	1.	**1.4**	-	2.0	**2.2**	-
Dnj2MN567713	2.0	**2.4**	-	1.2	**1.5**	-	1.0	**1.3**	-	2.0	**2.2**	-
DS2MN567716	2.3	**2.3**	-	1.4	**1.6**	-	1.4	**1.4**	-	2.5	**2.8**	-

3.4.4 Extraction of antibacterial activity by different solvents:

Following different solvents were used for extraction of antibiotics from four antibacterial actinomycetes:-

1- Acetone. 2- Chloroform. 3- α- Hexane, 4- Ethyl acetate. 5- Methanol. 6-Water.

Only one solvent (Ethyl acetate) mixed with supernatant could give two-layer. Bioassay of these solvents was tested for bioactivity to select the best solvent for extraction. When water was used as solvent, inhibition zone was observed (2.8 cm) for isolate *Streptomyces levis* JH1MN135857 against *B.subtilis*, (1.9cm) against *P. aeruginosa*, (1.8cm) against *E.coli*, (2.4cm) against *S. aureus* (Fig. 8).

Ethyl acetate was the best solvent, which gave maximum activity for all isolates against both Gram positive and Gram-negative bacteria. For ethyl acetate maximum inhibition zone was (3.7cm) against *B. subtilis*, (2.8cm) against *S. aureus*, (2.1cm) against *E. coli* and (2.6cm) against *P.aeruginosa* (Fig. 9). The best solvent used for all isolates was ethyl acetate because maximum activity was observed with crude antibacterial was dissolved completely in this solvent for all four antibacterials.

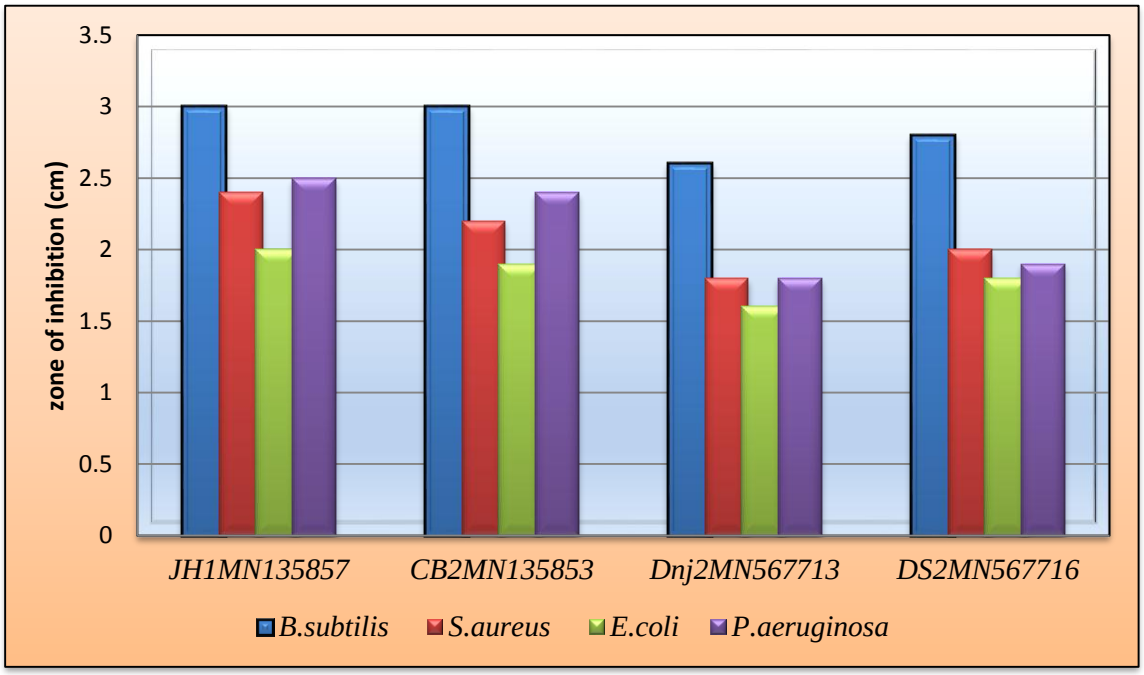


Fig. 8: Inhibition zone of the antibacterial extracts by water

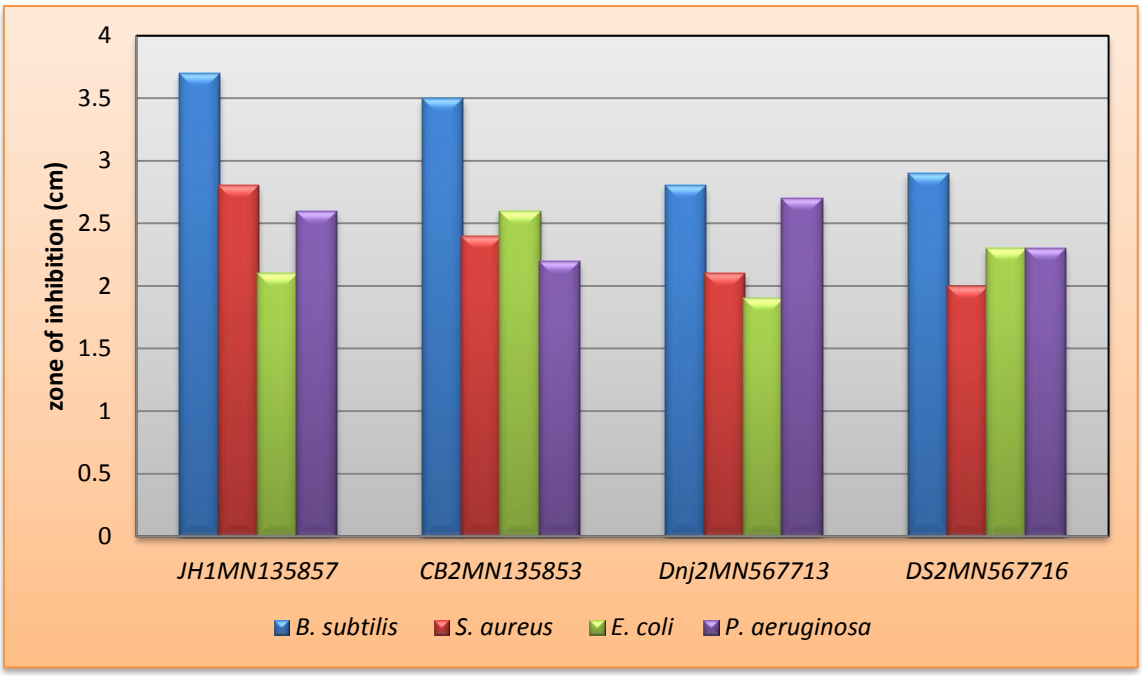


Fig. 9 Inhibition zone of the antibacterials extracts by ethyl acetate.

3.4.5 Purification by column chromatography:

Purification of four antibacterial was done by column chromatography in 15 fractions and each fraction was tested for bioactive compounds. Isolate *Streptomyces levis* JH1MN135857 gave the maximum inhibition in fraction no. 8 against *B.subtilis* (3.9 cm), *S. aureus* (3 cm), *E. coli* and (2.6cm) against *P. aeruginosa* (2.8 cm).

Isolate *Streptomyces thermocarboxydus* CB2MN135853 gave maximum inhibition zone in the fraction no.19 against *B. subtilis* (3.6 cm), *S. aureus* (2.9 cm) *E. coli* (2.4 cm) and (2.9 cm) against *P. aeruginosa*.

Isolate *Actinoplane digitatis* Dnj2MN567713 gave maximum inhibition zone in the fraction no. 4 against *B. subtilis* (2.5 cm), *S. aureus* (2.2 cm) *E. coli* (2.1 cm) and (2.9 cm) against *P.aeruginosa*.

The maximum inhibition zone was found with the fraction no.11 was 3.1cm for isolated *Nocardia kroppenstedtii* DS2MN567716 against *B. subtilis*, (2.2 cm) *S. aureus*, (2.5 cm) *E. coli* and (2.6 cm) against *P. aeruginosa*.

Table 4: Characterization of antibacterial fractions of actinomycetes.

Actinomycetes isolates	Color of antibiotic residue	Fraction. No.*	Antibacterials zone (cm)			
			B. subtilis	***P. aeruginosa***	***S. aureus***	***E. coli***
Streptomyces levis JH1 MN135857	Light Yellow	8	3.9	2.8	3	**2.3**
Streptomyces thermocarboxydus CB2 MN135853	Yellow	19	3.6	2.9	2.9	**2.4**
Actinoplane digitatis Dnj2MN567713	Light yellow	4	2.5	2.9	2.2	**2.1**
Nocardia kroppenstedtii DS2MN567716	Red orange	11	3.1	2.6	2.2	**2.5**

*Fraction by silica gel column chromatography.

3.4.6 Physicochemical and biological properties of antibacterials:

3.4.6.1 Physical properties of antibacterials:

Three antibacterials have oily appearance Dnj2MN567713 and JH1MN135857 and DS2MN567716 but CB2MN135853 is a powder. There were two antibacterials Dnj2MN567713 and JH1 MN135857gave light yellow color, but CB2MN135853 gave yellow. Antibacterial DS2MN567716 color is reddish-orange. The melting point of the antibacterial Dnj2MN567713 was 226℃, antibacterial JH1 MN135857 was 235℃, antibacterial CB2 MN135853 was 192℃ and antibacterial DS2MN567716 was 213℃. Solubility on different solvents of four antibacterials were same Dnj2MN567713, JH1MN135857, DS2MN567716, and CB2MN135853 which were soluble completely in polar solvents such as ethyl acetate, water and slightly soluble in chloroform, α-hexane and soluble in acetone and methanol.

3.4.6.2 Biological properties of antibacterials:

The effect of four antibacterials activity of Dnj2MN567713, JH1 MN135857, *CB2 MN135853* and DS2MN567716 against tested pathogenic bacteria using Minimum Inhibitory Concentration (MIC) as the lowest concentration of an antimicrobial to prevent the visible growth of bacteria and Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium. The results of *in vitro* antibacterial activity by purified compound of isolates Dnj2MN567713, JH1 MN135857, CB2MN13585 and DS2MN567716 at five concentrations (1000, 500, 250, 125, and 62.5 µg/ml) against Gram-positive (*B. subtilis, S. aureus*)

and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) by broth dilution technique are summarized in Table 27. All the tested antibacterials inhibited the bacterial pathogens and significant differences were observed among most of the samples. As antibacterials JH1 MN135857 and CB2 MN135853 were highly effective, the MIC and MBC against the tested organisms were determined. The MIC was 187.5 µg/ml for *B. subtilis*, 187.5 µg/ml for *E. coli*, and 93.75 µg/ml for *P. aeruginosa* while it was 375 µg/ml for *S.aureus*. The MBC was found to be 250 µg/ml against *B.subtilis, E. coli* while *P. aeruginosa* 125 µg/ml and 500 µg/ml for *S. aureus*. The MIC of antibacterial Dnj2MN567713 was 750 µg/ml for *S. aureus, E. coli* while it was 375 µg/ml for *B. subtilis as* well as 187.7 µg/ml for *P.aeruginosa.* The MBC was found to be 1000 µg/ml *S.aureus, E. coli while* it was 500 µg/ml for *B. subtilis* as well as 250 µg/ml *P.aeruginosa.* The MIC of antibacterial DS2MN567716 was 375µg/ml for *B.subtilis, S.aureus,* while *P.aeruginosa* and *E. coli* were 750 µg/ml**.** The MBC was found to be 500 µg/ml *B.subtilis, S. aureus,* while *P. aeruginosa* and *E. coli* were 1000 µg/ml.

The mean value of MIC for JH1 MN135857, CB2 MN135853 DS2MN567716 and Dnj2MN567713 were 210.9375 µg/ml, 210.9375 µg/ml, 234.375 µg/ml, 562.5 µg/ml and 515.625 µg/ml respectively, and the mean value of MBC for JH1, CB2, DS-2 and Dnj-2 were 281.25 µg/ml, 281.25 µg/ml, 750 µg/ml and 687.5 µg/ml respectively. Therefore, JH1 MN135857 and CB2 MN135853 were highly effective against pathogenic bacteria more then, DS2MN567716 and Dnj2MN567713.

3.4.6.3 Chemical properties of antibacterial JH1MN135857 and CB2MN13585

3.4.6.3.1 Antibacterial JH1MN135857:

Streptomyces levis JH1 MN135857. Spore chains were loop, hook, flexible and end of spore chains were fragmented. Aerial mycelia were grey and substrate mycelium was brown. The diffusible pigment was produced. The strain was positive for catalase production, nitrite reduction and hydrogen sulfide was not produced. The isolate utilized glycine, L-asparagine, L-tyrosine, and ammonium sulphate as a nitrogen source. The isolate utilized l -arabinose, D-fructose, D-glucose mannitol, lactose, citric acid, and lactic acid as a carbon source. The isolate utilized chitin, pectin, lecithin, and keratin. This isolate produced antimicrobial against targets, *B. subtilis S. aureus, E. coli,* and *P. aeruginosa.*

All these activities make this isolate very important and antibacterial produced by this isolate is important. The processes used to characterize and identify this antibacterial are as follows:

❖ **Thin Layer Chromatography (TLC):**

Thin layer chromatography study with purified antibacterial showed one spot with Rf value 0.89, these fractions showed maximum activity against *B. subtilis, S. aureus, E. coli, and P. aeruginosa.*

❖ **Gas Chromatography Spectrum(GCS):**

Analysis of this compound by GCMSMS give molecular formula as $C_{35}H_{59}NO_{11}$, and molecular weight as 670.4158. The compound name is Oleandomycin. From library of GCMS the known compound similar to antibacterial JH1MN135857 was found but not same compound and give the indication that this antibacterial is mostly new (Fig. 10).

❖ **Fourier Transform Infrared Spectrum (FTIRS):**

This analysis was used to detect the functional groups in this compound. The IR absorption at 3332.2 cm^{-1} is due to the presence of hydroxyl group (-OH) and at the absorption at 1647.5 cm^{-1} is due to the presence of stretching vibrations (C=C). The absorption at 2981.9 cm^{-1} is due to the presence of C-H group other peaks are 3734.8, 3649.1, 3589.4, 2888, 2322.1, 2132,950, 1017.6, and 113.8 cm^{-1} . Methanol was used as the solvent (Fig. 11).

❖ **UV Spectrum (UVS):**

The ultraviolet (UV) absorption spectrum of the isolated compound recorded absorption bands at 268. Absorbance within UV range confirmed the presence of unsaturation in the compound. (Fig. 12)

❖ **H-NMR Spectrum:**

Analysis of this compound by H-NMR gave molecular formula as $C_{35}H_{59}NO_{11}$ and molecular weight as 670.4185. (Fig. 13).

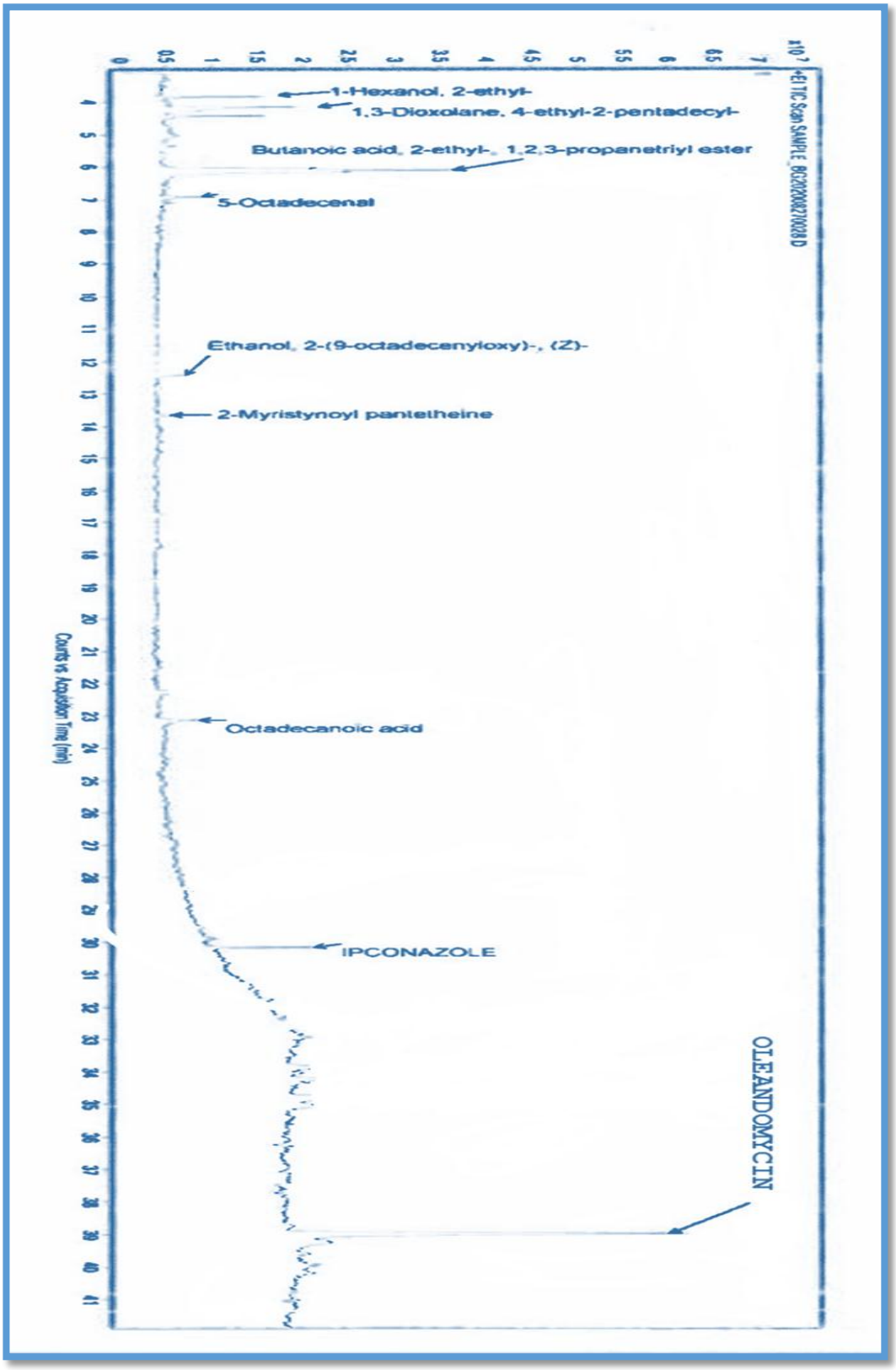


Fig. 10: Peak analysis by Gas Chromatography Mass Spectrum of the antibacterial JH1 MN135857 used methanol as solvent.

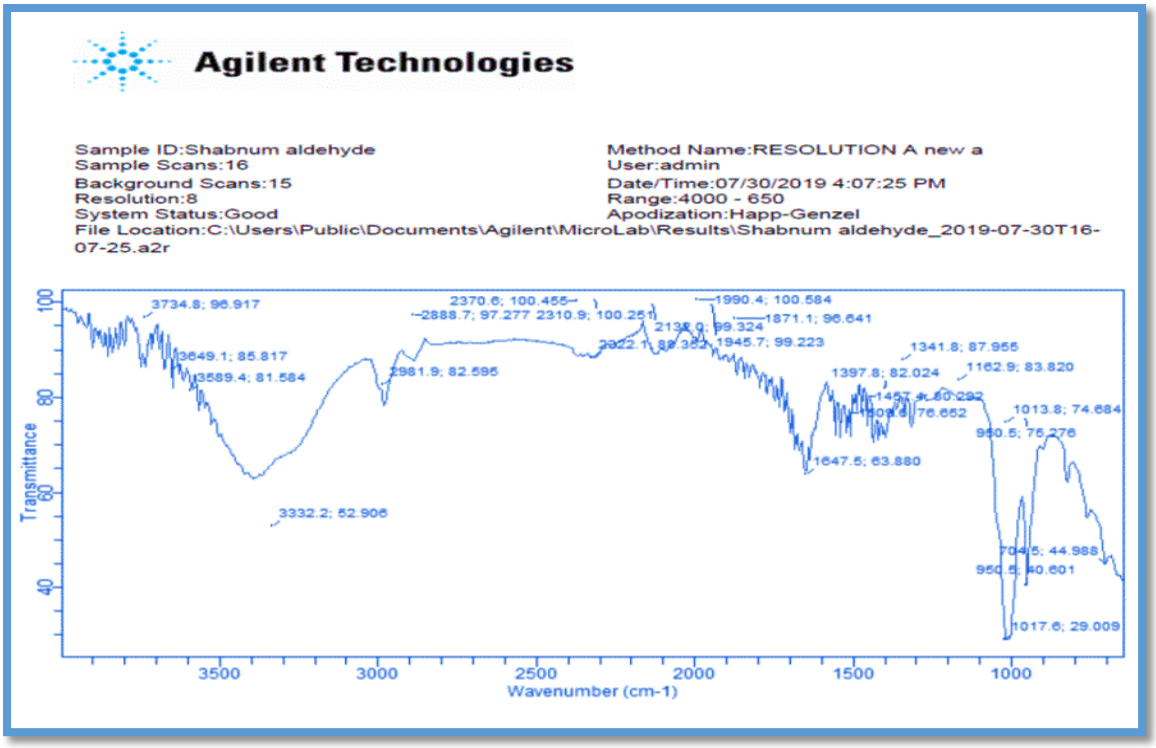


Fig. 11: Antibacterial JH1 MN135857 by IR Spectrum (FTIR) of the antibacterial used methanol as solvent.

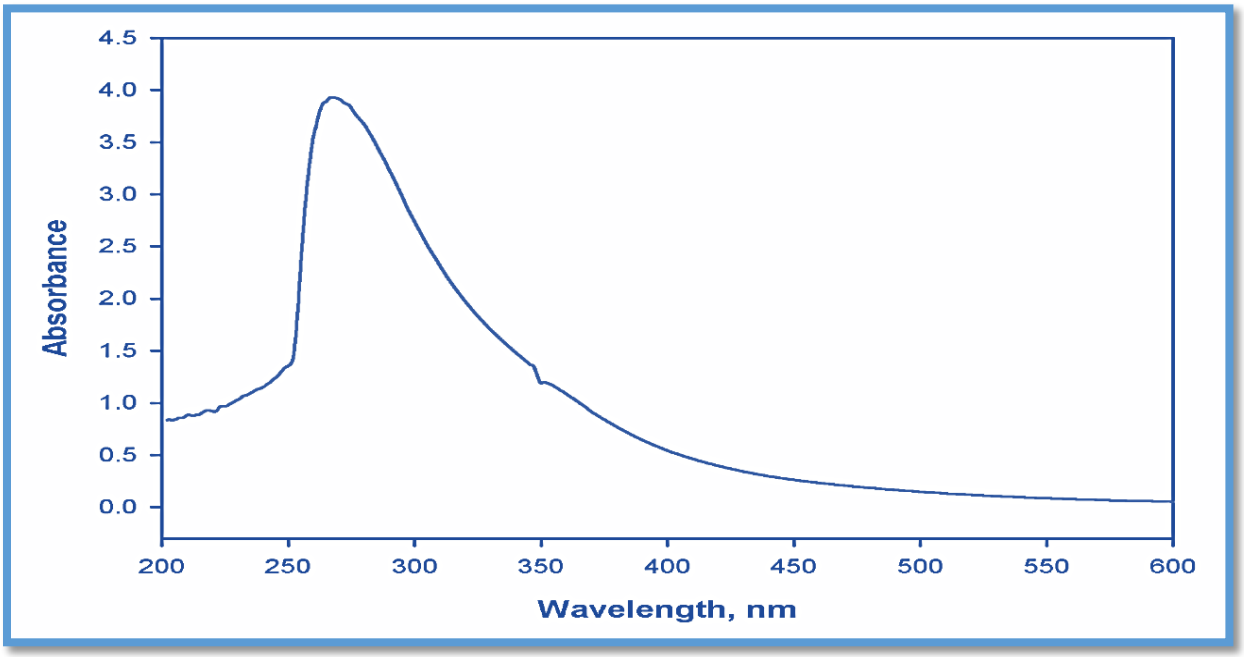


Fig. 12: Peak report by UV Spectrum of the antibacterial JH1 MN135857 used methanol as solvent

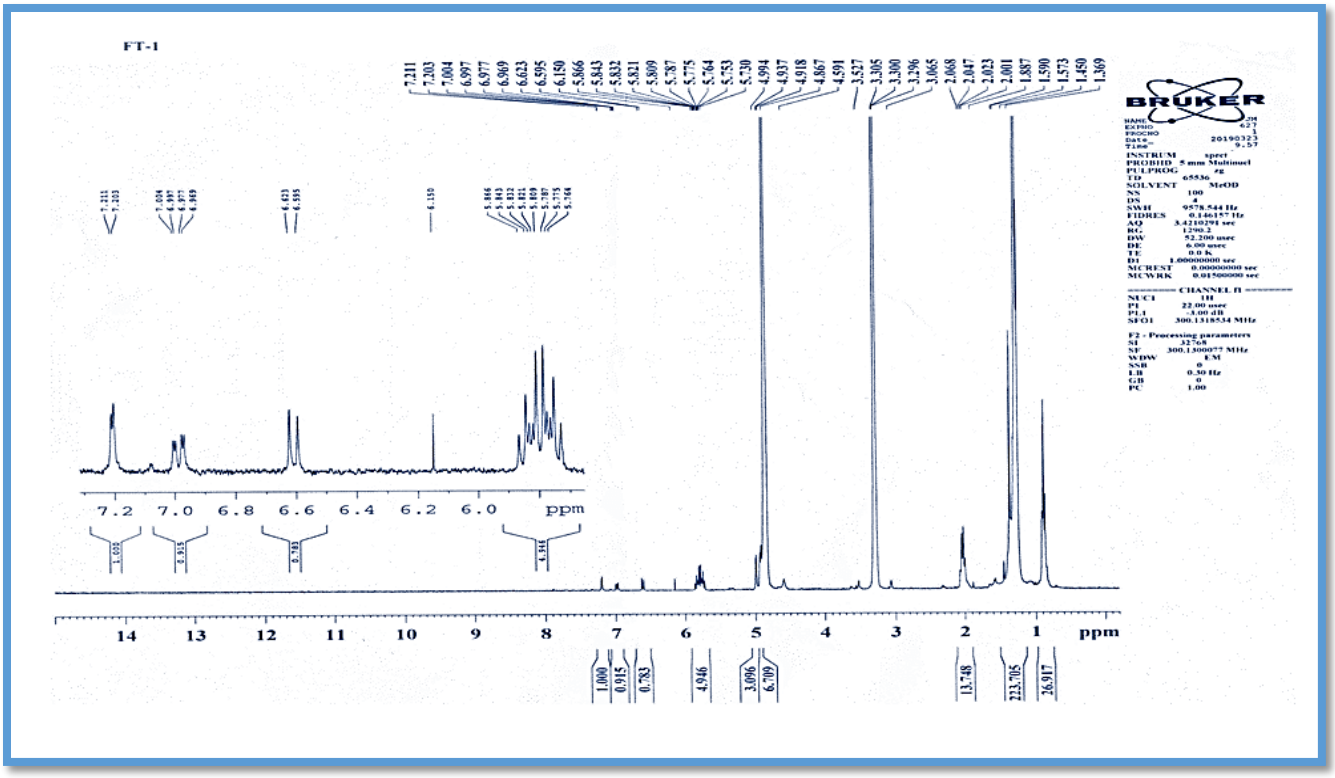


Fig. 5: H-NMR of the purified compound (antibacterial JH1 MN135857).

Table 5: Chemical characters and physico-chemical properties of the antibacterial JH1 MN135857

Chemical characters	**characters**
Molecular formula	$C_{35}H_{59}NO_{11}$
Formula weight	670.4185
Composition	C = 61.31, H = 8.61, N = 2.04 and O = 28.02
physico-chemical properties	**properties**
Appearance:	Yellow
Melting point	235
UV	268
IR V_{max} (cm^{-1})	3332.2, 1647.5, 2981.9,3734.8, 3649.1, 3589.4, 2888, 2322.1, 2132,950, 1017.6, 113.8
Rf value	0.89

3.4.6.3.2 Antibacterial CB2 MN135853

Streptomyces thermocarboxydus CB2 MN135853. Spore chains were straight. Aerial mycelia were white and substrate mycelium was yellow. The diffusible pigment was produced. The strain was positive for nitrite reduction, and hydrogen sulfide. The isolate utilized glycine, L-asparagine, L-tyrosine, and ammonium sulphate as a nitrogen source. The isolate utilized D-fructose, D-glucose, mannitol, lactose, citric acid, and lactic acid as a carbon source. The isolate utilized chitin, pectin, lecithin, and gelatin. This isolate produced antimicrobial against targets, *B. subtilis S. aureus, E. coli,* and *P. aeruginosa.*

All these activities make this isolate very important and antibacterial produced by this isolate is important. The processes used to characterize and identify this antibacterial are as follows:

❖ **Thin Layer Chromatography (TLC):**

Thin layer chromatography study with purified antibacterial showed one spot with Rf value 0.50, these fractions showed maximum activity against *B. subtilis S. aureus, E. coli, P. aeruginosa*.

❖ **Gas Chromatography Spectrum(GCS):**

Analysis of this compound by GCMSMS give molecular formula as $C_{37}H_{67}NO_{13}$, and molecular weight as 733.92548. The compound name is Erythromycin. From library of GCMS the known compound similar to antibacterial CB2 MN135853 was found but not same compound and give the indication that this antibiotic is mostly new (Fig. 14).

❖ **Fourier Transform Infrared Spectrum (FTIRS):**

This analysis was used to detect the functional groups in this compound. The IR absorption at 1726.16 cm^{-1} is due to the presence of hydroxyl group (-OH) and at the absorption at 1464.42 cm^{-1} is due to the presence of stretching vibrations (C=C). The absorption at 1401.29 cm^{-1} is due to the presence of C-H group other peaks are 1401.29, 1380.19, 1342.48, 1314.15, 1264.67, 1245.06, 1167.94,1120.58,1090.71,1079.43,1054.90,998.17,9,53.55,908.41,895.51,865.6,831.95,804.34,789.83,746.73,724.86,632.31,614.56,594.12,580.67,548.92,518.40, 495.13, 471.93, 458.48, and 422.3 cm^{1}. Methanol was used as the solvent (Fig. 15).

❖ **13C and 1H-NMR Spectrum:**

Analysis of this compound by 13C and 1H-NMR gave molecular formula as $C_{37}H_{67}NO_{13}$ and molecular weight as 733.9. (Fig 16 and 17).

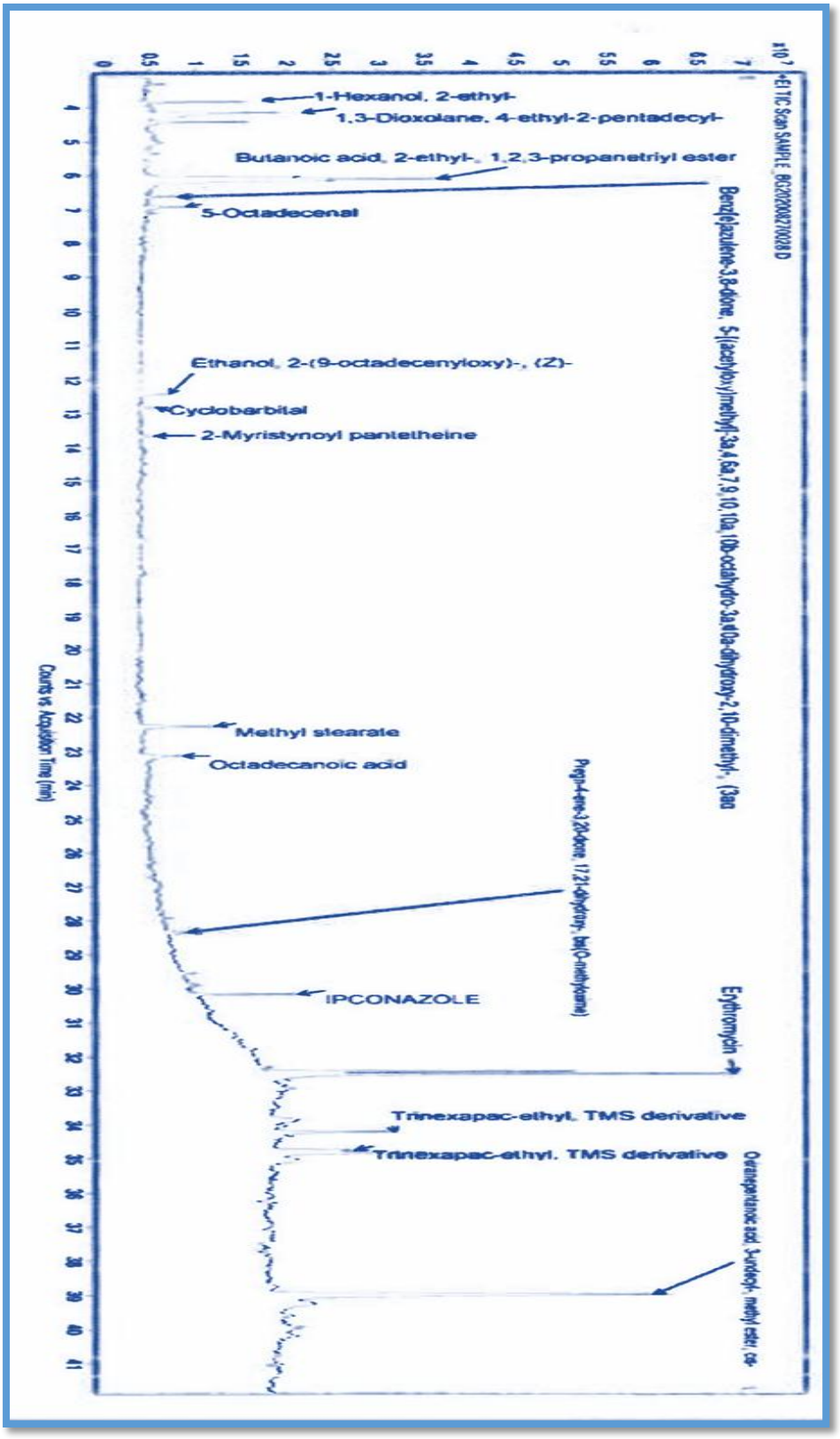


Fig. 14: Peak analysis by Gas Chromatography Mass Spectrum of the antibiotic CB2 MN135853 used methanol as solvent.

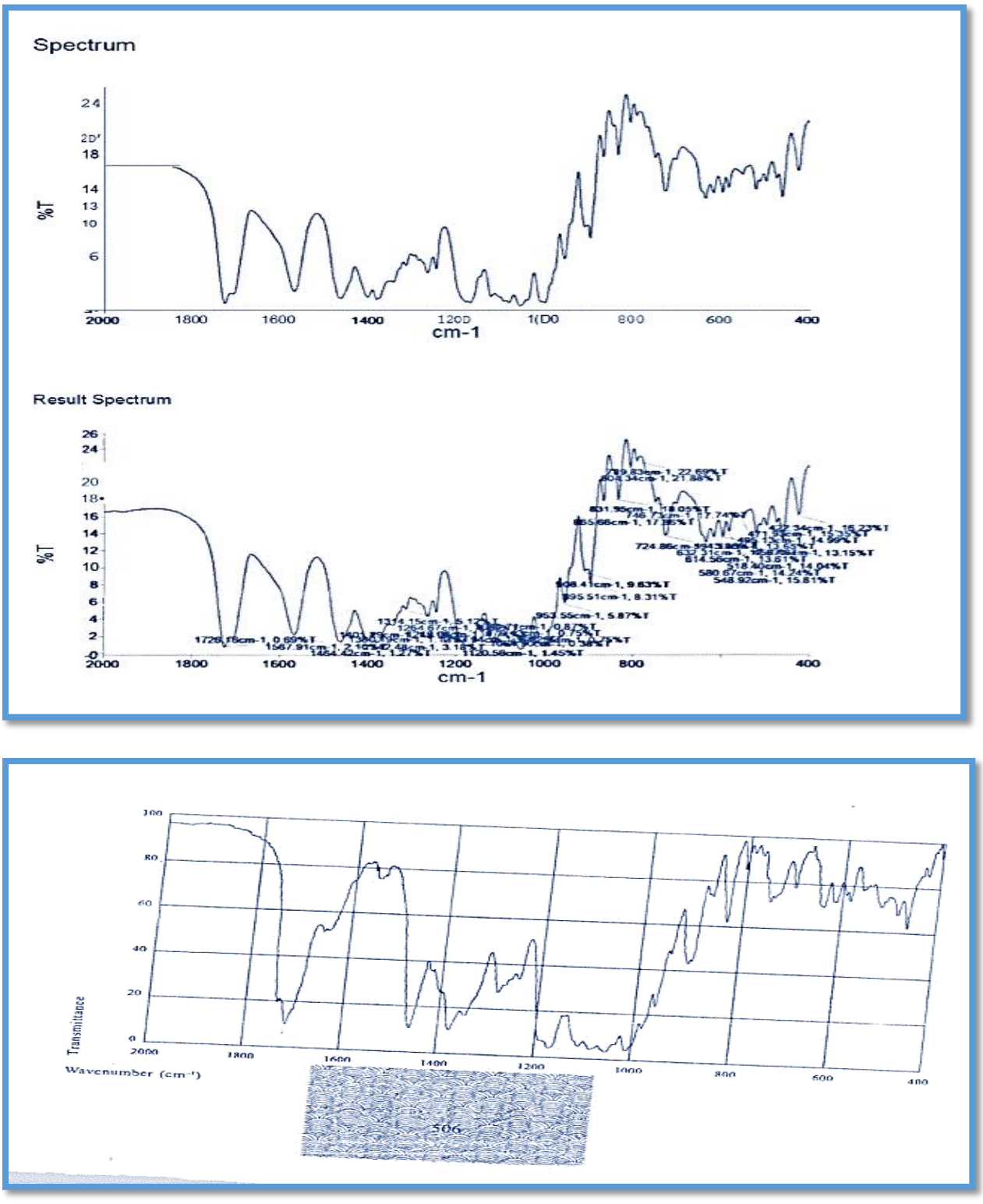


Fig. 15: Antibacterial CB2 MN135853 by IR Spectrum (FTIR) of the antibacterial used methanol as solvent.

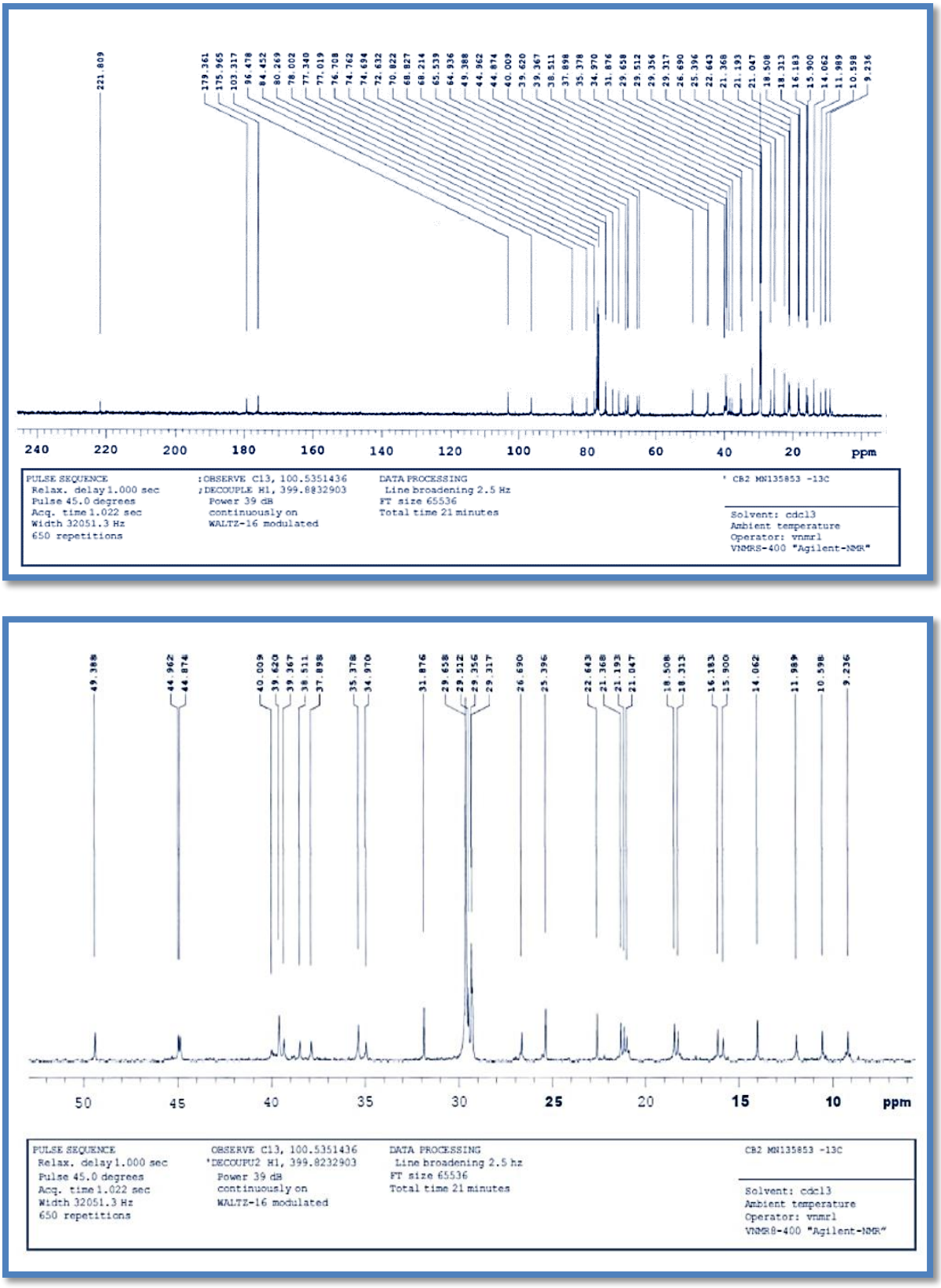


Fig 16: C-NMR of the purified compound (antibacterial CB2 MN135853).

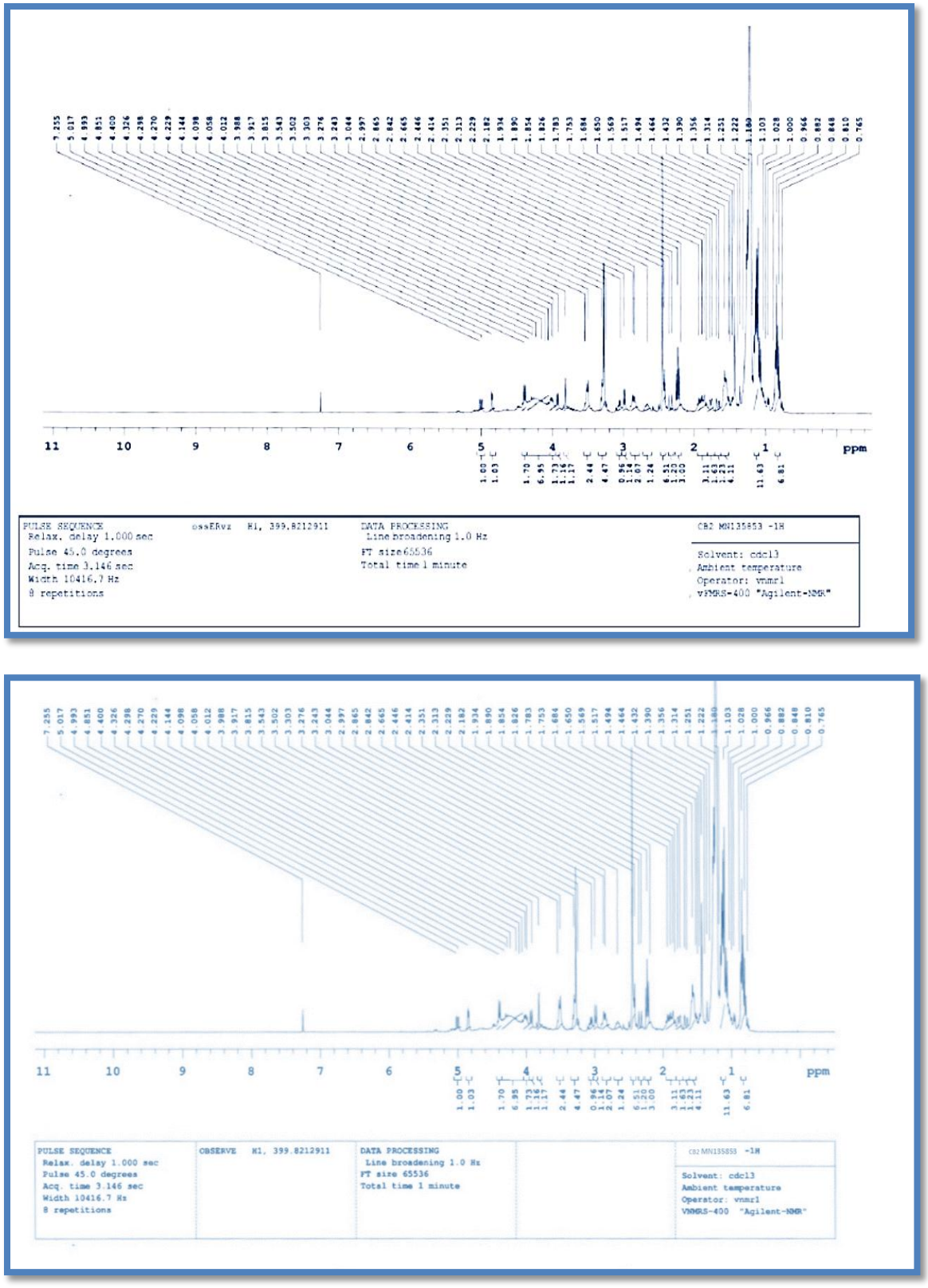


Fig 17: H-NMR of the purified compound (antibacterial CB2 MN135853).

Continuation of Fig.:17

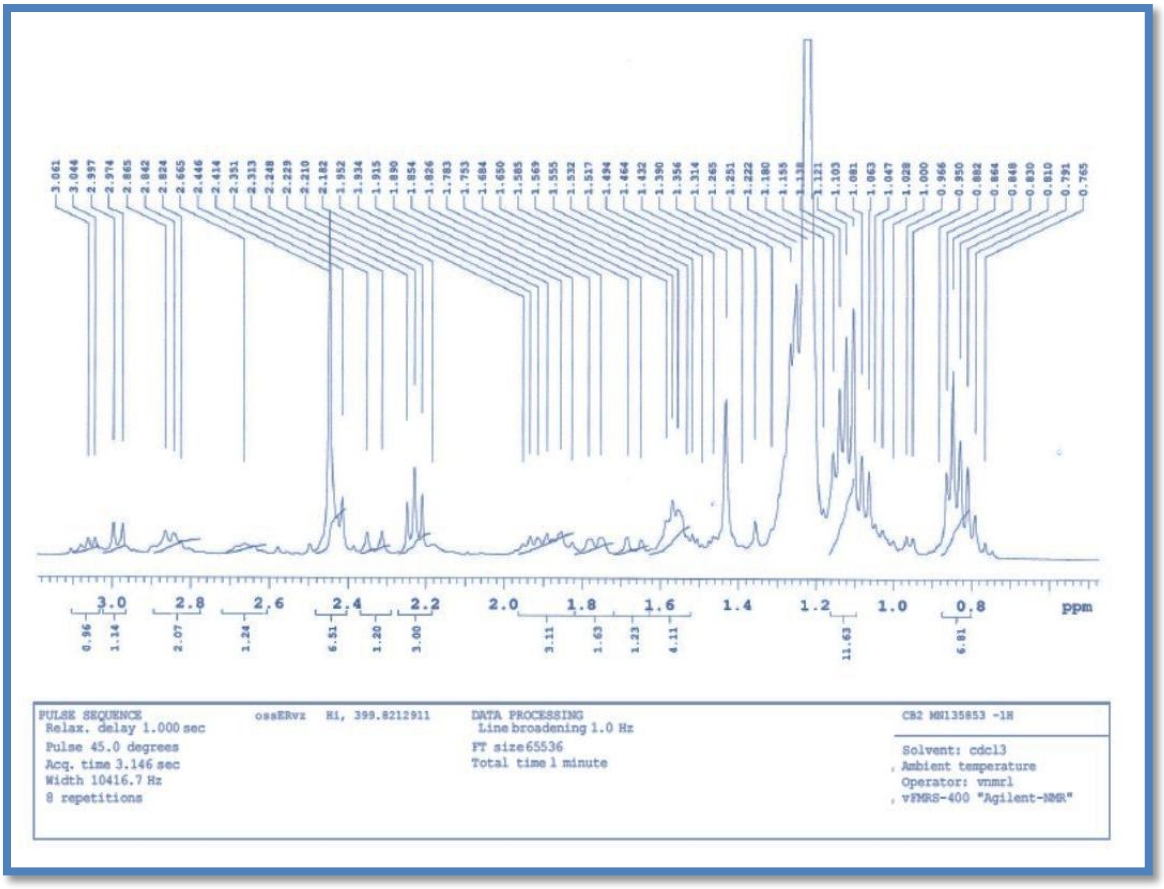


Table 6: Chemical characters and physico-chemical properties of the antibacterial CB2 MN135853

Chemical characters	**Characters**
Molecular formula	$C_{37}H_{67}NO_{13}$
Formula weight	733.92548
Composition	C = 61., H = 9.2, N.= 1.90 and O = 28.33
physico-chemical properties	**properties**
Appearance:	Yellow
Melting point	192
IR V_{max} (cm^{-1})	1726.16,1464.42,1401.29,1380.19,1342.48,1314.15,1264.67,1245.06,1167.94,1120.58,1090.71,1079.43,1054.90,998.17,9,53.55,908.41,895.51,865.6,831.95,804.34,789.83,746.73,724.86,632.31,614.56,594.12,580.67,548.92,518.40, 495.13, 471.93, 458.48, and 422.3
Rf value	0.58

CHAPTER FOUR

SUMMARY AND CONCLUSION

SUMMARY

In summary, actinomycetes are admitting as a root of commercially important products, e.g. antibiotics, enzymes, enzyme inhibitors, immune modifiers, and vitamins. Most of the actinomycetes are soil-borne bacteria and that plays an important role in the mineralization process. However, actinomycete metabolites or derivatives thereof account for approximately two-thirds of the naturally occurring antibiotics. These bacteria have been the focus of aggressive research efforts since the discovery of actinomycin in 1940 from actinomycete by Selman Waksman at Rutgers University**.** Actinomycetes are Gram-positive bacteria belonging to the order Actinomycetales, characterized by the formation of the substrate and aerial mycelium on solid media, presence of spores and a high G+C content of the DNA (60-70 mol%). Actinomycete strains are assigned to taxa based on cultural and morphological characteristics, physiological characteristics, staining properties, the presence or absence of spores, the capacity to grow on different carbon sources, nitrogen sources, NaCl tolerance, pH, and temperature.

In this study, soil samples were collected from three districts of Hadhramout state of Yemen viz., Sah district, Ghayl Bin Yamin district, and Ash-Shihr district to investigate the diversity of actinomycetes. Actinomycetes isolated on selective media were characterized and identified. The culture supernatants were screened for biological activities. This research was aimed at establishing primary and rapid screening systems for detecting antibacterial antibiotics. This screening system was utilized to look for novel antibacterial antibiotics from novel actinomycetes. The identification method that we used allowed unknown, non-Streptomyces soil isolates to be identified to a genus or small sub-group of genera.

The present study consisted of four major aspects. The idea of carrying out this work was to get the diversity of actinomycetes, from dams, caves and mountains of Hadhramout Yemen. The objectives of the research work undertaken are given below:

1. Isolation of actinomycetes from some dams, caves and mountain soils of Hadhramout –Yemen.
2. Screening of actinomycetes for antibacterial and enzymatic activity.
3. Identification of actinomycetes, which have a strong antibacterial activity.
4. Study on the production, purification and characterization of potent antibacterial compound.

Actinomycetes are common in terrestrial habitats such as dams, caves, mountains, agricultural fields and forest soils where they form major constituents of the actinomycete community. In

contrast, members of this group grow within pH range 6.5 to 10.0 with an optimum around 7.0 and temperature from 20℃ – 45℃ with an optimum around 37 ℃

About 19 soil samples from 19 locations in three districts viz., Sah, Ghayl Bin Yamin and Ash-Shihr were analysed over a period of six months for physicochemical parameters and actinomycetes population. The physicochemical analysis included temperature, pH, and nature of the soil samples. Isolation and enumeration of actinomycetes performed by soil dilution plate technique using Starch Casein Agar (SCA) of pH 7.0, incubated for 7 days at 37 ºC. The isolated colonies were maintained on starch casein agar slants.

In the present study, 219 actinobacteria colonies from 18 different locations of the terrestrial and aquatic ecosystems were isolated. Among the 219 colonies, 103 isolates were morphologically different. The maximum isolates were taken from Ghayl bin Yamin district soil followed by Sah and Ash-Shihr.

Antimicrobial activity of all the isolates grown on solid as well as in liquid media was studied. Production of antimicrobials in solid media was tested by agar disc diffusion method (ADD) and in liquid media by well agar diffusion technique against *Bacillus subtilis ,Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa.* Out of 103 actinomycete isolates, only 26 isolates showed antibacterial activity by both techniques and three of them were strong antibacterial. viz., *Nocardia kroppenstedtii* (Ds-2), *Streptomyces levis* (JH-1), *Actinoplane digitatis* (Dnj-2), and *Streptomycetes thermocarboxydus CB2 MN135853* which possessed activity against all the pathogens tested, were selected for further studies.

The antibacterial isolates (26) were screened for nine enzyme activities viz., amylase, caseinase, chitinase, gelatinase, keratinase, lipase, urease, pectinase, and lecithinase. It was found that all the isolates produced urease and 81% of them produced amylase, lecithinase, and caseinase, the isolates producing pectinase were 69%, keratinase 73%, gelatinase and chitinase 77%, lipase 39%, and urease 92%. Thus, the actinomycete isolates produced a wide range of enzymes. Such organisms producing diverse enzymes must be playing an important role in the mineralization of organic matter in the habitats.

The taxonomy of actinomycetes was based on the morphological, physiological, biochemical properties and molecular genome (16S rRNA). All strains were assigned to groups based on colony and micro-morphology, staining properties, the presence, and absence of substrate mycelium and spores, the ability to grow on different carbon and nitrogen sources or in presence of inhibitory

compounds and antibiotics. Microscopic, cultural, biochemical and other criteria were used to identify actinomycete isolates.

Out of 26 antibacterial actinomycete isolates, 20 were identified as *Streptomyces* and four isolates were identified as *Nocardia*, two isolates were identified as *Actinoplane*, some of these isolates might be new strains.

The molecular characteristics like 16S rRNA gene sequence analysis evolutionary analysis, 16S rRNA gene secondary structure prediction, restriction. The 16S rRNA gene sequences of all isolates were deposited in the GenBank Accession numbers. The all isolated was identified as: *Streptomyces thermocarboxydus CB2 MN135853, Streptomyces variabilis DJ-1 MN1357855,* Streptomyces gobitricini *CS3 MN135852, Streptomyces lilaceus CT2 MN135854, Streptomyces rochei DW3 MN135856, Streptomyces rochei DAB11 MN567711 Streptomyces luridiscabiei Dab3 MN559792, Streptomyces levis JH1 MN135857, Streptomyces carpaticus Dsh-2 MN559789, Streptomyces enissocaesilis HBT3 MN559788, Streptomyces plicatus SBO3 MN559790, Streptomyces alfalfae DJ3 MN559791, Streptomyces sp CS2 MN567712, Streptomyces sp CM2 MN559793, Actinoplanes digitatis Dnj2 MN567713, Nocardia asteroids C.Lah3 MN567714, Nocardia otitidiscaviarum JH7 MN567715, and Nocardia kroppenstedtii DS2 MN567716.*

All 26 antibacterial actinomycete isolates showed strong antibacterial activity, of which four isolates were selected for further studies. Fermentation parameters for antibiotic production were determined. The time course of antibiotics production was also studied. The growth curve of *Streptomycetes levis JH1 MN135857* and *Streptomycetes thermocarboxydus CB2 MN135853* were recorded, started to produce antibiotics after 3 days and maximum of antibiotic production after the 8^{th} day of incubation. On the other hand, the growth curve of the *Nocardia kroppenstedtii DS2MN567716,* and *Actinoplane digitatis Dnj2MN567713* were recorded, started to produce antibiotics after 7 days of incubation and maximum antibiotic production was between 14-21days of incubation.

Most of *Streptomyces* species grew faster than other genera and reach to stationary stage *Streptomyces levis JH1* MN135857and *Streptomyces thermocarboxydus CB2 MN135853* grew faster than other genera and reach stationary stage on the 8th day of incubation at OD 0.580 and 0.600 at A 540 respectively. As well as, the maximum growth OD 0.482 at A540 for isolate *Nocardia kroppenstedtii DS2MN567716* and minimum growth OD 0.342 at A540 for isolate *Actinoplane digitatis Dnj2MN567713*

Antibiotic obtained from *Streptomyces levis JH1* MN135857and *Streptomyces thermocarboxydus CB2 MN135853* were selected for further studies. The antibiotic obtained was purified using TLC. The characteristics of the antibacterial compound of *Streptomyces levis JH1 MN135857* are given below: light yellow, viscous in nature, and its melting point was 235°C. It was stable at pH 6-7 and temperature 30-45°C. Ultimately, based on the UV, FT-IR and HNMR spectral studies, the antimicrobial compounds of *Streptomyces levis* JH1 MN135857 exhibited conjugated double bonds, hydroxyl group, stretching vibration group and ethyl group. Molecular formula was $C_{35}H_{59}NO_{11}$, with molecular weight 670.4185. Finally, the characteristics of the antibacterial compound of *Streptomyces thermocarboxydus CB2 MN135853* are given below: yellowish, viscous in nature, and its melting point was 192°C. It was stable at pH 6-7 and temperature 30-45°C. Ultimately, based on the FT-IR and 13C 1H NMR spectral studies, the antimicrobial compounds of *Streptomyces thermocarboxydus CB2 MN135853* exhibited conjugated double bonds, hydroxyl group, stretching vibration group and ethyl group. Molecular formula was $C_{37}H_{67}NO_{13}$, with molecular weight 733.92548

Conclusion

Based on the outstanding findings of the study made on actino-bacteria, the doctoral thesis concludes as follows:

1. Distribution of actinobacteria was influenced by physico-chemical parameters such as the soil type, soil color, pH and temperature.
2. The Agar Disc Diffusion method (ADD) was more effective for antibacterial activity than Agar Well Diffusion method (AWD) against bacterial pathogen.
3. All actinomycete isolates that were selected for further study belonged to *Streptomyces* genus, *Actinoplanes* genus and *Nocardia* genus.
4. Almost isolated are new strains
5. *Streptomyces* have strong antibacterial activity more than *Nocardia* and *Actinoplane* against both Gram-positive and Gram-negative pathogenic bacteria.
6. 16S r RNA amplification using universal eubacterial primers: 27 F and 1492R yielded a single amplicon of approximately 1500 bp for all the actinomycete isolates.
7. The most abundant species in soil isolated accurately belongs to *Streptomyces* (77%) followed by *Nocardia* (16.54%), and *Actinoplane* (7).
8. Most of actinomycetes isolates had enzymatic activity such as caseinase, amylase, lipase, lecithinase, keratinase, chitinase, gelatinase, catalase and urease.
9. Most of all of the actinomycetes were resistant to most of the antibiotics and sensitive to gentamycin ($10\mu g$), polymyxin- B (100 units) kanamycin ($30\mu g$), tetracyclin ($30\mu g$), and novobiocin ($30\mu g$).
10. Starch casein medium was the best media for production of antibiotic.
11. The pH 7 was the best pH for production of antibiotic.
12. The 37℃ was the best temperature for production of antibiotic.
13. Ethyl acetate was the best solvent used for extraction of antibiotic.
14. Antibacterial with molecular formula $C_{35}H_{59}NO_{11}$, and molecular weight 670.4185 Da. isolated from *Streptomyces levis* JH1 MN135857.
15. Antibacterial with molecular formula $C_{37}H_{67}NO_{13}$, and molecular weight 733.92548Da. isolated from *Streptomyces thermocarboxydus CB2 MN135853*

Scope for future research:

1. Expanding source of actinomycetes by carrying out ecological assessments of environments other than terrestrial soils.
2. Modified medium for isolation of non -Streptomyces is required to discover more novel actinomycetes to produce novel antibiotics.
3. Screening of metabolites from representatives of *Nocardia* and *Actinoplane* for novel, antifungal and antitumor activity.
4. Process optimization for the production and downstream processing of antibacterial from non- *Streptomyces* isolates
5. Testing the potential of all isolated to control the palm weevil “Rhynchophorus ferrugineus."

CHAPTER FIVE

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5 References

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PUBLICATIONS

4 Publications

1. **Ali Mohammed Abdullah Bawazir**, Ahmed Al-Haddad, Abdullah AL- Mahdi, Manjula Shantaram*. "Actinomycetes from Mountains of Hadhramout- Yemen". International **Journal of Current Microbiology and Applied Sciences (IJCMAS)** 2017; Vol. 6, Issue 8: 3521-3530. ISSN: 2319-7692 (Print), 2319-7706(Online).

2. **Ali Mohammed Abdullah Bawazir** and Manjula Shantaram*. "Non-streptomyces and antibiotics from special habitats– a review" **Biomedicine** 2017; 37(4): 473-478. ISSN: 0970 2067. Impact Factor: 0.02.

3. **Ali Mohammed Abdullah Bawazir** and Manjula Shantaram*. "Antibacterial Activity of Actinomycetes Isolated from Different Habitats of Hadhramout- Yemen" **Haya – Saudi Journal of Life Sciences.** January 2018; 3(1): 5-9. ISSN 2415-6221 (Online) ISSN 2415-623X (Print). IF = 0.36. 2017.

4. **Ali Mohammed Abdullah Bawazir**, Gunashree B. S., Manjula Shantaram*. "Isolation and Characterization of Nocardia from Laiha Cave Soil in Hadhramout of Yemen as a Source of Antibiotic" **International Journal of Science and Research Methodology (IJSRM),** 2018;9(3):121-130. ISSN: 2454-2008.

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6. **Ali Mohammed Abdullah Bawazir** and Manjula Shantaram*. "Ecology and Distribution of Actinomycetes in Nature- A Review". **International Journal of Current Research (IJCR)** July 2018;10(7): 71664-71668. ISSN: 0975-833X. SJIF 2018= 7.749.

7. **Ali Mohammed Abdullah Bawazir,** Palaksha, and Manjula Shantaram*. "An Implication of Actinomycetes for the Human well-being: A Review". **International Journal of Pharmacy and Pharmaceutical Sciences,** 2019;11(5): 11-18. ISSN- 0975-1491.

5 Presentations in conferences:

❖ Poster

1. Ali Mohammed Abdullah Bawazir and Manjula Shantaram, (2017) "a comparative inhibition study of actinomycetes against pathogenic bacteria using agar disc diffusion and agar well diffusion methods" national conference Mangalore University, Karnataka-India.
2. Ali Mohammed Abdullah Bawazir and Manjula Shantaram, Biodiversity and distribution of actinomycetes from Hadhramout- Yemen influenced by Physico-chemical properties of environment. National Conference on Trends in Bioactive Natural Products and Healthcare (BNPHC 2017), October 6th & 7th 2017, DOS in Biochemistry, Mangalore University, Post Graduate Centre, Chikka Aluvara, Kodagu, Karnataka-571232 India.

❖ Oral

1. Ali Mohammed Abdullah Bawazir and Manjula Shantaram. Potency of actinomycetes against multidrug resistant bacteria. International Conference On concepts on the Role of Indian Medicine and Phytoceuticals in Maintenance of Health, 15-17th November 2018, DOS in Biochemistry, Mangalore University, Post Graduate Centre, Chikka Aluvara, Kodagu, Karnataka-571232 – India.
2. Ali Mohammed Abdullah Bawazir and Manjula Shantaram. The Enzyme Activity of Actinomycetes Isolated from Dam Soils. National Level Conference On "Resent Advances in Material Science" held on 15th February 2019 by Department of Chemistry, Field marshal K.M Cariappa College, Madikeri, Kodagu-571232.
3. Ali Mohammed Abdullah Bawazir and Manjula Shantaram. Antimicrobial Potential of *Nocardia kroppenstedtii* (DS-2) Isolated from Soil Dam of Hadhramout-Yemen. International Conference on Impact of Innovations in Science and Technology for Societal Development (IISTSD-2019) jointly organized by Kongunadu Arts and Science College and Faculty of Technology, Mahasarakham University, Thailand during 19th-21st Sept. 2019 will be held at Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.
4. Ali Mohammed Abdullah Bawazir and Manjula Shantaram. A study on bioactive actinomycetes isolated from Hadhramout, Yemen. National Conference on Phytochemicals and Microbial Bioactive Compounds - Role in Agriculture and Human welfare, 3 - 4 October, 2019, Department, of Microbiology and Biotechnology, Bangalore University, Bengaluru-560056, Karnataka, India.

Republic Of Yemen
Ministry of Agriculture & Irrigation
Hadhramout Gov. Office - Mukalla

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وزارة الزراعة والري
مكتب محافظة حضرموت - المكلا

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Date: التاريخ:

CERTIFICATE

This is to certify that **Mr. ALI MOHAMMED ABDULLAH BAWAZIR**, Nationality Yemeni, passport No. **07665034** is a bonafide full-time Ph.D. student, in Microbiology, Mangalore University, Karnataka, India. Registration No. (MU/EXB /Ph.D. /CR.50/ Micro/2020-21/E.13).

He has collected natural soil samples. As part of the requirements of his research work in India. We confirm that soils were natural and contain **NO PATHOGENIC MICROORGANISMS AND TOXINS**. There is **NO OBJECTION** to the exit of those soil samples from Yemen.

Therefore, we ask for your cooperation with the researcher to complete the requirements for award Ph.D. This was given at his request.

Awad Bakman

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The office of the Ministry in Hadhramout
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